A STUDY OF A NONSEPTATE MUTANT OF *SCHIZOPHYLLUM COMMUNE* AT THE CELLULAR LEVEL

A DISSERTATION

SUBMITTED TO THE FACULTY OF ATLANTA UNIVERSITY

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

BY

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ATLANTA, GEORGIA

MAY 1984

ABSTRACT

BIOLOGY

IKE, JUSTUS O. B.A., Dana College, 1975

M.S., Atlanta University, 1979

A Study of a Nonseptate Mutant of Schizophyllum Commune at the Cellular Level

Advisor: Dr. John Mayfield

Doctor of Philosophy degree conferred May 21, 1984

Dissertation dated May, 1984

In the tetrapolar basidiomycete Schizophyllum commune a mutation in strain 9004 (chocolate) impairs hyphal growth as well as brings about an accumulation of extracellular granules and nonseptate condition. A higher amount of residual glucose was observed in the growth medium of the chocolate mutant. Estimation of crude protein showed that the mutant contained more crude protein in its growth medium as compared to that of the wild-type.

Partial and complete hydrolysis of the mutant cell wall demonstrated that it is mainly composed of small amounts of chitin and two glucans called S and R-glucan, and that its S/R ratio is low. The incorporation of labeled uridine diphosphate-N-acetyl-D-glucosamine in cell-free extracts of the chocolate mutant of Schizophyllum commune showed very

low incorporation into insoluble chitin of the mutant. All of the above evidence suggest that there is lesion or weakness in the chocolate cell wall and this may be affecting the general physiology of the mutant.

An ultrastructural observation of the hyphae revealed a wider cell wall in the mutant as compared to that of the wild-type. Besides, the study showed that there is an increased number of vacuoles and intrusion in the subapical region. SEM and TEM exhibited cytoplasmic materials and exudate, respectively, which were secreted by the chocolate mutant.

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ACKNOWLEDGMENTS

I am indebted to Dr. John E. Mayfield (my major advisor) for his supervisory role in this investigation. His understanding and patience are highly appreciated. My sincere thanks go to the following advisors: Dr. Curtis Parker for his guidance in this project; Dr. Rosalyn Patterson for her advice in this investigation and her able leadership of the Atlanta University Biology Department. I am also grateful to the Atlanta University Biology Department and the Resource Center of Science and Engineering for sponsoring the investigation.

Gratitude is offered to my parents, Mr. and Mrs. Ike, my sisters, brothers and the other members of the Ike family for their inspiration, moral and financial support which made this study possible and the Doctor of Philosophy degree a reality.

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CHAPTER I

INTRODUCTION

Schizophyllum commune Fries is a wood-rotting basidiomycete with "gilled" fruit-bodies which is classified under Aphyllophorales (Donk, 1964). Among Hymenomycetes this species is the most extensively studied from a genetic point of view, with more emphasis on the sexual incompatibility system that controls morphogenesis (Kniep, 1920; Papazian, 1950; Raper, 1966; Koltin *et al.*, 1967; Raper *et al.*, 1960; Raper and Raper, 1973). Simultaneously, interest has developed in the structural and chemical aspects of hyphal morphogenesis (Mehadevan and Tatum, 1965; Paul *et al.*, 1968; Niederpruem and Wessels, 1969; Wessels, 1978).

The complex septal pore structure (dolipore septum), as described by Moore and McAlear (1962) has been considered one of the major characteristics of the higher basidiomycetes (Alexopoulus and Mims, 1979). This structure was first observed by Buller (1933). Since these earlier studies, septal structure has become an important morphological criterion in distinguishing basidiomycetes from ascomycetes. Ascomycetes produce simple cross walls that permit nuclei to pass from cell to cell along with other materials. While basidiomycete-type septum does appear to prohibit nuclear migration in dikaryotic hyphae, the dolipore septum has also been

reported to occur in other Basidiomycetes such as Polystictus versicolor (Girbardt, 1958), Coprinus stercorarius (Ellis et al., 1972), and Tremella sp. (Khan and Talbot, 1976).

Dolipore septa have been found in both homokaryotic and dikaryotic strains of S. commune (Jersild et al.,1967). They also observed simple septa among the common-A heterokaryons, the simple septa were also observed in a strain with secondary mutation at the B incompatibility locus (Wessels, 1971; Raudaskoski, 1972). The occurrence of aseptate hyphae is rare among basidiomycetes. Fungi having aseptate hyphae are generally believed to be confined to Phycomycetes.

The surface structure and cell wall of fungi increasingly attract the attention of biochemists and mycologists because these structural parts provide enough information about chemical and physical identity of the various wall components which make it possible to selectively remove or stain these components and determine their location in the wall by electron microscopy (Nickerson and Bartnicki-Garcia, 1964; Hackenbrock, 1966; Cooke, 1969; Casselton and Kirkham, 1975; Mollenhauer et al., 1978). The fungal cell wall has been studied from different aspects, such as biochemical, morphological and ultrastructural (Niederpruem and Hackett, 1961; Wang and Miles, 1966; Myron and Connelly, 1971; Raudaskoski, 1972; Yuh, 1974). The molecular mechanism

of cell wall formation in fungi begins to appear with increased frequency after the discovery of uridine diphosphate by Capatto and coworkers (1950).

This discovery by itself indicates the increasing importance of studies on the mechanism of cell wall structure, using the fungi as models for simple eucaryotic cells. Nevertheless, information emerging from this investigation would contribute to a better understanding of fungal cell wall, various surface-related biological phenomena, such as morphogenesis, drug resistance and others. Besides, it would also provide a good starting point for further ultrastructural or physiological work. Because of the great complexity of these problems, we will deal mainly with the functional aspects of chocolate mutant's cell wall and try to find their possible relationship to regulation of morphological development.

The objective of the present work is to investigate the physiological study of a nonseptate mutant of Schizophyllum commune at the cellular level. This particular organism is selected because it can serve as a model system for studying extensive migration of nuclei, mitochondria and perhaps vacuoles. It therefore appeared worthwhile to investigate this mutant. Data obtained in this investigation may prove useful as a basis for future biological problems, such as plasticity, cell communication, drug resistance, and others.

CHAPTER II

REVIEW OF LITERATURE

Schizophyllum commune is a heterothallic, tetrapolar fungus. Analyses of hyphal wall fractions of this organism have shown three distinct homopolymers: S-glucan, R-glucan and a relatively small amount of chitin (Wessels, 1965; Valk and Wessels, 1977; Wessels and Sietsma, 1979). S-glucan, an alkali-soluble glucan which belongs to a class of α-(1-3) linkages in the glucan, has been demonstrated in a variety of other fungi (Bacon *et al.*, 1968). R-glucan is an alkali-insoluble glucan with highly branched β-glucan with (1-3) and (1-6) linkages. Wessels (1965) pointed out that R-glucan in the cell wall of *Schizophyllum* resembles "yeast glucan" in that it is more resistant to laminarinases (Wessels, 1969).

Aitken and Niederpruem (1970) observed that the S-glucan/R-glucan ratio of ungerminated basidiospores was considerably less than unity whereas there was a marked shift in the ratio of S-glucan/R-glucan during germination. They pointed out that this increase in S/R value, during normal germination, reflects preferential synthesis of S-glucan or selective degradation of R-glucan during outgrowth. Archer and his associates (1977) and Wessels and Niederpruem (1967) clearly showed that degradation of R-glucan results

in a prominent increase in the S/R ratio of the cell wall components, but S-glucan biosynthesis is primarily responsible for the changes that occur during outgrowth.

Besides, the detection of S and R-glucan from S. commune's hyphal wall fraction by Wessels (1965); Lahoz et al., (1970); and Wessels and Sietsma (1979), a small amount of chitin was also detected. Chitin, a polysaccharide consisting of N-acetyl-glucosamine in β-1, 4 linkage, has long been considered to be a component of the fungal mycelial wall. "According to Foster (1949), since 1811, Braconnot identified chitin in fungi; many qualitative microchemical tests have been developed to prove the presence of chitin." Its biosynthesis in a cell-free system was first described by Glaser and Brown (1957), who found that an enzyme preparation from Neurospora crassa catalyzes the incorporation of N-acetyl-glucosamine units from uridine 5'diphosphate (UDP)-GlcNAC into a polymer indistinguishable from the authentic chitin. A single enzyme, UDP-2-acetamido-2-deoxy-D-glucose: chitin 4- β -acetamidodeoxyglucosyltransferase, known as chitin synthetase, seems to be involved in the reaction. However, McMurrough and coworkers (1971) reported that chitin synthetase is actually involved in the synthesis of chitin. Vries and Wessels (1975) demonstrated that in the presence of cycloheximide, protoplasts of S. commune produced chitin for about 5 hr at a rate similar to that of control protoplasts.

The polyoxins are antibiotics produced by Streptomyces cacoi varasoenis. They were discovered and developed in Japan, where they are now formulated into agricultural fungicides. The fungicidal activity of polyoxin D on Neurospora crassa was investigated by Endo and coworkers (1970). They observed that treated hyphae had a greatly reduced incorporation of [^{14}C]-glucosamine into chitin, and further, that the polyoxin was a competitive inhibitor of chitin synthetase. The binding efficiency of polyoxin D at the same site or sites as the substrate UDP-N-acetylglucosamine has been demonstrated by Dahne et al. (1976), Bartnicki-Garcia and Lippman (1972), and Schwarz et al. (1969). They compete at the same binding site or sites because the structures of polyoxin D, nikkomycin and UDP-N-acetylglucosamine are closely related (Dahne et al., 1976).

Gull (1978) observed in Basidiomycetes that the plasmalemma is continuous along both sides of the septum and around the dolipore swelling. He pointed out that the membrane of the parenthosome is a differentiated form of endoplasmic reticulum. He also emphasized that the septa protect the mycelium from mechanical damage or osmotic injury at the location of the growing tip. The exact region of septal disruption and the passage of nuclei through simple septa in a puff mutant was demonstrated by Mayfield (1974). His observation reveals that septal dissolution begins with parenthesome and pore swelling on one side of the

septum. This is then followed by erosion of the septum. He also observed that migration is accompanied by a flow of other cytoplasmic organelles leaving behind highly vacuolated hyphae.

The nature of hyphal branching patterns has commonly been investigated in order to better understand fungal morphology. One approach to this problem is the study of morphological abnormalities obtained through genetically altered strains or by the addition of fungal metabolites. The term paramorphogen was coined by Tatum et al. (1949) to refer to substances that alter wild-type morphology. Cellobiose is one of the paramorphogens that increases branching frequency of S. commune. This agent is also responsible for S. commune's dense and restricted growth (Wilson and Niederpruem, 1974).

The hyphal cell wall and branching patterns were not the only structural aspects of S. commune that some investigators have looked into. Herbert and Niederpruem (1964), Thornton (1968), and Robinson et al., (1969), however, observed the cytoplasmic membrane, granules, endoplasmic reticulum, mitochondria and vacuoles during an ultrastructural study of S. commune basidiospores.

CHAPTER III

MATERIALS AND METHODS

Cultures and Media

Wild-type strain 699 and the mutant strain 9004 of *Schizophyllum commune* were obtained from Dr. P. G. Miles, Department of Biology, State University of New York at Buffalo, New York, and grown on complete agar medium containing 20g D-glucose, 2g Bacto peptone, 0.5g $MgSO_4 \cdot 7H_2O$, 1g K_2HPO_4, 0.46g KH_2PO_4, 20g agar and one liter of distilled water. During physiological studies, cultures of these organisms were grown in a complete liquid medium as above except for the omission of agar. All medium ingredients were autoclaved together in 250ml Erlenmeyer flasks containing 50ml of the medium with initial pH of 6.8. The cultures were incubated at room temperature for 16 days on a Lab-Line orbit Environ-Shaker 18 (Model 627) gyrating at 120 rpm.

Dry Weight Determination

In order to compare the growth of wild-type strain 699 with mutant strain 9004, both organisms were grown 16 days in the above medium and harvested at 4 day intervals. The growth was determined by studying changes in dry weight of the mycelium. Dry weight values were established by filtering the mycelium through a Buechner funnel with Whatman No. 1 filter paper. The mycelium was then placed in preweighed aluminum weighing pans and dried overnight at 120 C

in a hot air oven. Measurement of pH of the culture filtrate was also performed using a Beckman pH meter model 140A.

Residual Glucose Determination

Residual glucose was determined by the modified method of Hyvarinen and Nikkila (1962) in which 5 ml of O-toluidine reagent was added to 0.1 ml of the growth medium. The mixture was placed in a vigorously boiling water bath for exactly 10 min. After 10 min the test tube was quickly removed, placed in tap water for approximately 3 min and the color measured at maximal absorption 620 nm. This procedure was carried out at 4 day intervals for 16 days.

Crude Protein Determination

Mycelia of the wild-type strain 699 and mutant strain 9004 were harvested, washed and homogenized with glass homogenizer in 1 ml of cold (4-5 C) 0.05 M Tris buffer (pH 7.4). An additional 3 ml of 0.05 M Tris buffer was used to transfer the homogenate into centrifuge tubes. Cellular debris was removed by centrifuging the mixture at 15000 rpm for 30 min. The supernatant fluid was used for protein estimation.

Crude protein determination was established by a modified method of Bradford (1976). Coomassie Brilliant blue G-250 was purchased from Sigma Chemical Company. Ten grams of Coomassie Brilliant blue G-250 was dissolved in 5 ml of 95% ethanol. To this solution, a mixture of 10 ml of phosphoric acid and 70 ml of distilled water was added. This

solution was diluted to a final volume of 100 ml with distilled water. The mixture was vacuum filtered 3 times with single filter paper and 2 times with double filter paper. Five milliliters of this protein reagent was added to 0.1 ml of cellular crude protein and the contents mixed with a vortex mixer. After 2 min at room temperature the absorbance was measured with Gilford spectrophotometer, model 240, set at 595 nm. Similarly, 5 ml of protein reagent was added to 0.1 ml of culture filtrate and the absorbance was also measured as described above.

Determination of N-acetylglucosamine

Isolation of the Cell Wall. Mycelia of wild-type strain 699 and mutant strain 9004 were grown in liquid medium as previously described. The cell wall was isolated according to the modified method of Siehr (1976). Mycelia of the organism were harvested, washed and stored frozen. The mycelium of each strain was thawed and homogenized in chilled 0.8% NaCl for 1 min in a Waring blender and centrifuged. Between each mechanical treatment the cell walls were centrifuged, and resuspended in 10% sucrose followed by centrifugation and resuspension in 8% NaCl. Finally, a chilled mortar and pestle were used to break the cell walls. In this manner, a preparation was obtained in which all the mycelia were broken on both sides of any septa, as determined by staining a portion of the cell wall with 0.25% methylene blue and observing the specimen under a light

microscope. The specimen was extracted with petroleum ether in a soxhlet apparatus for 10 hr to remove lipids. Finally the cell wall preparation was air-dried, ground with a chilled mortar and pestle to a fine powder.

Determination of Glucosamine. Ten milliliters of distilled water was added to 50 mg of cell wall preparation, and 10 mg of chitinase. To this mixture, 1 mg of cycloheximide was added, and the suspension was incubated for 48 hr at room temperature.

Glucosamine was assayed according to the modified method of Good and Bessman (1964). One milliliter of aqueous solution of hexosamine was poured into a test tube containing 0.2 ml acetic anhydride solution, and 1.0 ml of borate buffer was added. The test tube was sealed with aluminum foil and placed in a boiling water bath for 3 min, and cooled in an ice bath for 5 min. Then, dimethylaminobenzaldehyde was added to make a total volume of 10 ml. The mixture was incubated in a 37 C water bath for 20 min before the spectrophotometric reading was taken at 570 nm.

Hydrolysis of S-glucan. Fifty milligrams of cell wall material was dissolved in 3 ml of dilute HCl. The mixture was heated in boiling water for 10 min, and then treated with 3 ml of 5% potassium hydroxide (Schaefer, 1977). The suspension was incubated in a shaker at 25 C for 17 hr. After the incubation period, the suspension was centrifuged and the alkaline extract was adjusted to pH 4.5 with glacial

acetic acid. Hydrolysis of S-glucan was indicated by the presence of glucose which was determined by O-toluidine reagent.

Hydrolysis of R-glucan. The hydrolysis of R-glucan was carried out by dissolving 50 mg of cell wall material in 3 ml of dilute H_2SO_4 (Wessels, 1965). The suspension was heated in boiling water for 90 min, and centrifuged to remove the insoluble fraction. The supernatant was neutralized with solid $BaCO_3$, and the insoluble salts removed by centrifugation and filtration. The clear filtrate was heated and adjusted to pH 6.0 with dilute H_2SO_4, and R-glucan was assayed by the O-toluidine reagent method as described above.

Incorporation of Uridine Diphosphate [^{14}C]-N-acetylglucosamine into the Cell-free Insoluble Chitin of Strain 9004. Mycelium was harvested from liquid culture, washed twice in distilled water, and homogenized in the presence of 3 volumes of ice-cold 0.05 M Tris buffer, pH 7.5; containing 0.01 M $MgCl_2$ and 0.001 M EDTA (Glaser and Brown, 1957). A portion of the homogenized cell wall was stained with 0.25% methylene blue and observed with a light microscope to determine whether most of the cell walls were broken. After homogenization, the preparation was centrifuged at 2,000 g for 10 min to remove cell debris. The supernatant fraction was then centrifuged at 10,000 g for 20 min. The cell-free extract collected as a pellet was

used for the incorporation of uridine diphosphate-[^{14}C]-N-acetylglucosamine (UDP-[^{14}C]GlcNAc) (Jaworski et al., 1965). Forty milligrams of cell-free extract was suspended in 12 ml of the above buffer and kept frozen at -20 C for 2 days. Protein determinations were made by the method of Bradford (1976).

The reaction mixture for the incorporation contained 0.12 ml of UDP-[^{14}C]GlcNAc (Spec. Act. 51.7 mCi/mmol), 1.2 ml of N-acetyl-D-glucosamine, 0.1 ml of adenosine triphosphate, 0.6 ml magnesium chloride, 40 mg cell-free extract and 3 ml of potassium phosphate buffer, pH 6.0, in a final volume of 6 ml. The mixture was incubated for 2 hr at room temperature. The reaction was stopped by the addition of 0.5 ml of 3N perchloric acid. The mixture was centrifuged at 140,000 g for 30 min and washed three times with 2 ml of 0.3N perchloric acid ($HClO_4$) and once with 2 ml of distilled water (Glaser and Brown, 1957). The incorporation of UDP-[^{14}C]GlcNAc was determined by suspending the residue in 15 ml of PPO basis toluene. All measurements of incorporated radioactivity were made using a Beckman liquid scintillation counter model LS 7500.

Incorporation of UDP[^{14}C]GlcNAc into the Cell Walls of Strain 9004. In order to determine the incorporation of UDP[^{14}C]GlcNAc into intact (in vivo incorporation) hyphae, a modified method of Gooday (1971) was used. All the solutions used in this experiment were mixed in test tubes.

The incubation mixture contained 0.12 ml of UDP-[^{14}C]GlcNAc (Spec. Act. 51.7 mCi/mmol), 1.2 ml of N-acetyl-D-glucosamine, 0.1 ml ATP, 0.6 ml of $MgCl_2$ and 3 ml of potassium phosphate buffer, pH 6.0. To this, 1.255 ml of the incubation mixture was added into 5 ml Erlenmeyer flasks containing 40 mg of mycelia of strain 9004 and incubated for 2 hr at room temperature. The procedure was repeated with strain 699, heat shocked strain 699 and 9004.

The reaction was stopped by the addition of 0.17 ml of 3N perchloric acid, centrifuged at 140,000 g for 30 min and washed 3 times with 2 ml of 0.3N perchloric acid and once with 2 ml of distilled water.

The mycelium was homogenized in the presence of 1 ml of ice-cold 0.05 M Tris buffer, pH 7.5, containing 0.01 M $MgCl_2$ and 0.001 M EDTA. The incorporation of UDP-[^{14}C]GlcNAc was determined by suspending the insoluble chitin in 15 ml of 2,5 diphenyloxazole (PPO) basis toluene and counted as described above.

Phase Contrast Microscopy

A small triangle of cellophane membrane with attached hyphae at the edge was cut and inverted in a drop of distilled water on a microscope slide. The cellophane was floated from the mycelial mat, and a cover glass was used to cover the mycelium. This provided a very thin preparation in which the morphology of the hyphae was clearly visible. The preparation was viewed through a microscope and photographs were taken using a 35 mm camera.

Scanning Electron Microscopy

In order to study the hyphae external morphology of the wild-type strain 699 and mutant strain 9004, scanning electron microscopy (SEM) was used. The organisms were grown for six days on sterilized strips of cellophane membrane laid over on semi-solid agar medium (Snider and Raper, 1958) containing 20 g D-glucose, 2 g Bacto pepton, 0.5 mg $MgSO_4 \cdot 7H^2O$, 1 g K_2HPO_4, 0.46 g KH_2PO_4, 20 g agar and one liter of distilled water. Some pieces of the cellophane membrane were cut out and fixed as follows: some cellophane pieces were placed in cacodylate buffered 3% glutaraldehyde for 15 min at room temperature, transferred to an ice bucket and fixed for an additional 15 min. The hyphae were post fixed in cold 2% osmium tetraoxide in 0.05 M cacodylate buffer (pH 7.4) for 2 hr and washed for 1 hr in cold water with at least ten changes. They were rinsed in three changes of distilled water for 15 min each. After washing, they were dehydrated through a graded series of ethanol - 50%, 70%, 95% for 10 min each and two changes in 100% acetone for 10 min each.

The hyphae were also passed through a graded series of amyl acetate - 50%, 70%, 95% and 100% for 7 min, respectively. The specimens were dried in critical point, coated with carbon and then examined in Perkin-Elmer ETEC scanning electron microscope OS121.

Transmission Electron Microscopy

Pieces of cellophane membrane, with adhering hyphae of strain 9004 were fixed with 3% glutaraldehyde and post-fixed with osmium tetroxide. Material was kept overnight in 0.5% uranyl acetate, dehydrated in an ethanol series - 50%, 70%, 95% and 100% for 10 min each, and 2 changes of 100% acetone. The cells were infiltrated for 30 min in a mixture of acetone of Spurr's (1969) medium (1:1), followed by embedding in Spurr's low viscosity resin. The resin was polymerized in BEEM capsules at 65 C for at least 18 hr. Thin sections were stained with 0.5% uranyl acetate and lead citrate (Reynolds, 1963) and viewed with the RCA EMU-4 electron microscope operating with an accelerating voltage of 50 or 100 Kv.

CHAPTER IV

EXPERIMENTAL RESULTS

Dry Weight Determination of the Wild-Type and Mutant of S. commune in Shaker Culture

In order to gain some information as to the growth of the mutant strain 9004 as compared to wild-type strain 699, dry weight determinations were made at 4-day intervals. The dry weight of the wild-type mycelium showed a rather sharp increase during the first four days of incubation to 220 mg compared to only a 10 mg increase in the mutant's mycelium for the same period (Table 1). In the case of the wild-type mycelium, there is a sharp increase in dry weight for the first eight days with growth almost reaching a peak when the dry weight obtains a value of 130 mg. The mutant mycelium expresses a lag during the first 4 days without any appreciable increase occurring between 4 and 8 days. The peak dry weight is obtained 4 days earlier for the wild-type mycelium. At the end of the 16-day growth period, the mutant's dry weight is only 25% (110 mg) of that observed for the wild-type mycelium (440 mg).

The growth of filamentous fungi is also accompanied by a reduction in the pH of the growth medium. The initial pH of the liquid complete medium for both wild-type and chocolate strain was 6.8 (Table 1). The reduction of the pH in chocolate's growth medium was more rapid than that

Table 1. Dry weight and pH measurements of wild-type and chocolate mutant

Growth period in days	Wild-type Dry Weight in mg[a]	pH[a]	Chocolate mutant Dry Weight in mg[a]	pH[a]
0	0	6.8	0	6.8
4	220	6.5	10	6.0
8	350	5.5	90	4.8
12	430	5.2	110	4.4
16	440	5.1	110	4.4

[a]Average of three determinations

observed in the wild-type (Fig. 1). The pH at the end of the 16-day incubation period was 4.4 in the chocolate as compared to 5.1 in wild-type growth medium.

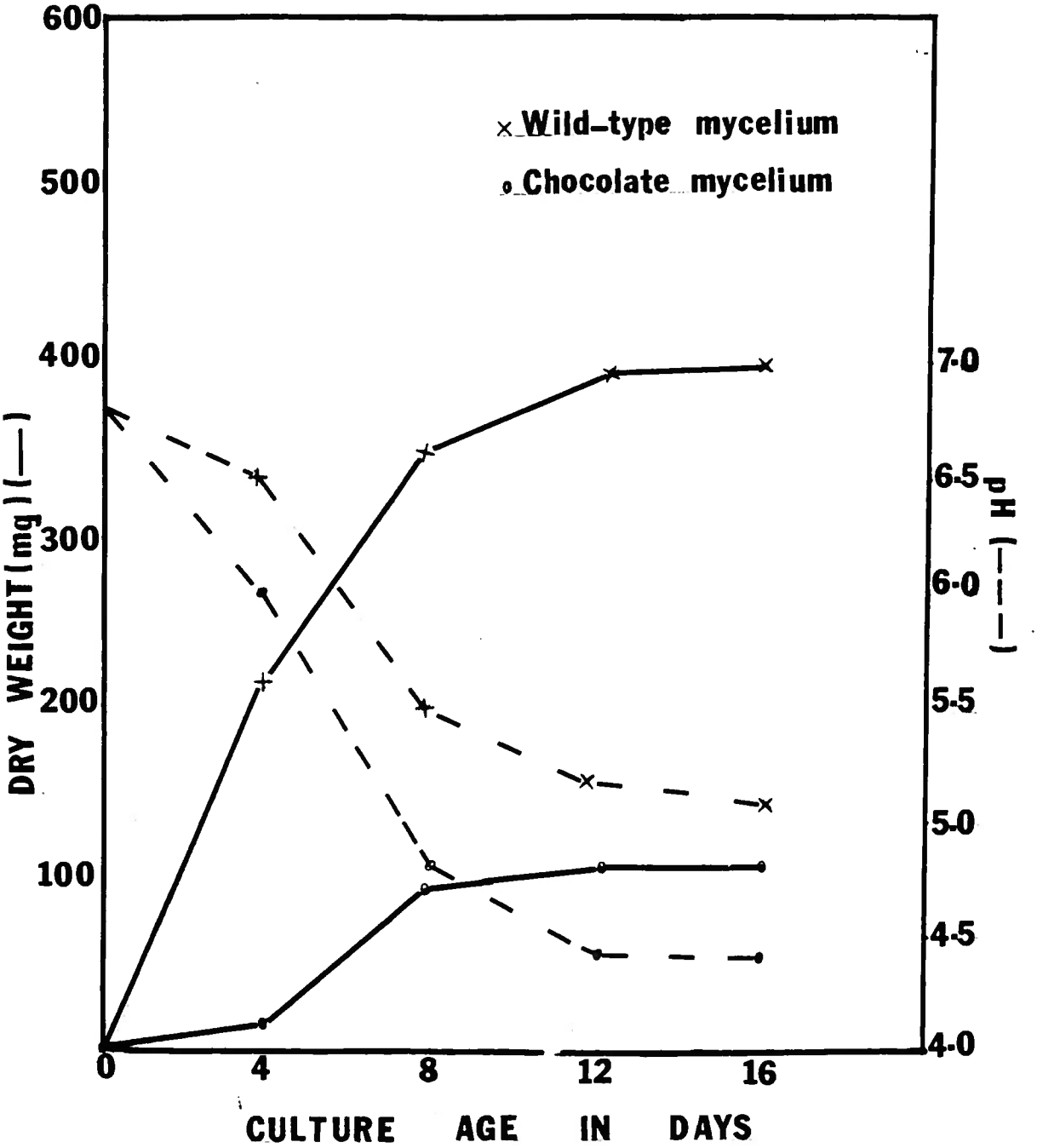
Residual Glucose Determination

Since glucose efficiency is associated with growth and the general physiological state of fungal organisms, the residual glucose levels determined for both chocolate and wild-type culture filtrate is most informative. The residual glucose in the growth medium of which the wild-type was grown showed that there was a significant reduction of glucose during the first 4 days. After four days there were 8.2 mg glucose/ml of medium in the wild-type compared to that of the mutant which was 14.0 mg/ml (Table 2). The residual glucose in the wild-type culture filtrate after 8 days was 5.0 mg/ml compared to 9.1 mg/ml observed in the culture filtrate of the mutant. After 12 days of incubation the residual glucose in the wild-type culture filtrate was 3.3 mg/ml while that of the mutant was 8.0 mg/ml (Table 2). The lowest residual glucose for both strains was observed at the end of the 16 day incubation period. At this time, the lowest residual glucose in the culture filtrate of the wild-type was 2.1 mg/ml whereas that of the mutant was 7.2 mg/ml (Fig. 2).

Crude Protein Content

To relate the growth and general physiology of the chocolate mutant, the crude protein was determined, since dry

Fig. 1. Growth and pH determinations of wild-type and chocolate mutant cultured in complete liquid medium on a shaker.


600
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5·0
4·5
4·0
× Wild-type mycelium
o Chocolate mycelium
DRY WEIGHT (mg) (——)
(— — —) pH
CULTURE AGE IN DAYS

Fig. 1.

Table 2. Residual glucose in complete medium

Growth period in days	Wild-type Glucose mg/ml[a]	Chocolate mutant Glucose mg/ml[a]
0	20.0	20.0
4	8.2	14.0
8	5.0	9.1
12	3.3	8.0
16	2.1	7.2

[a]Average of three experiments

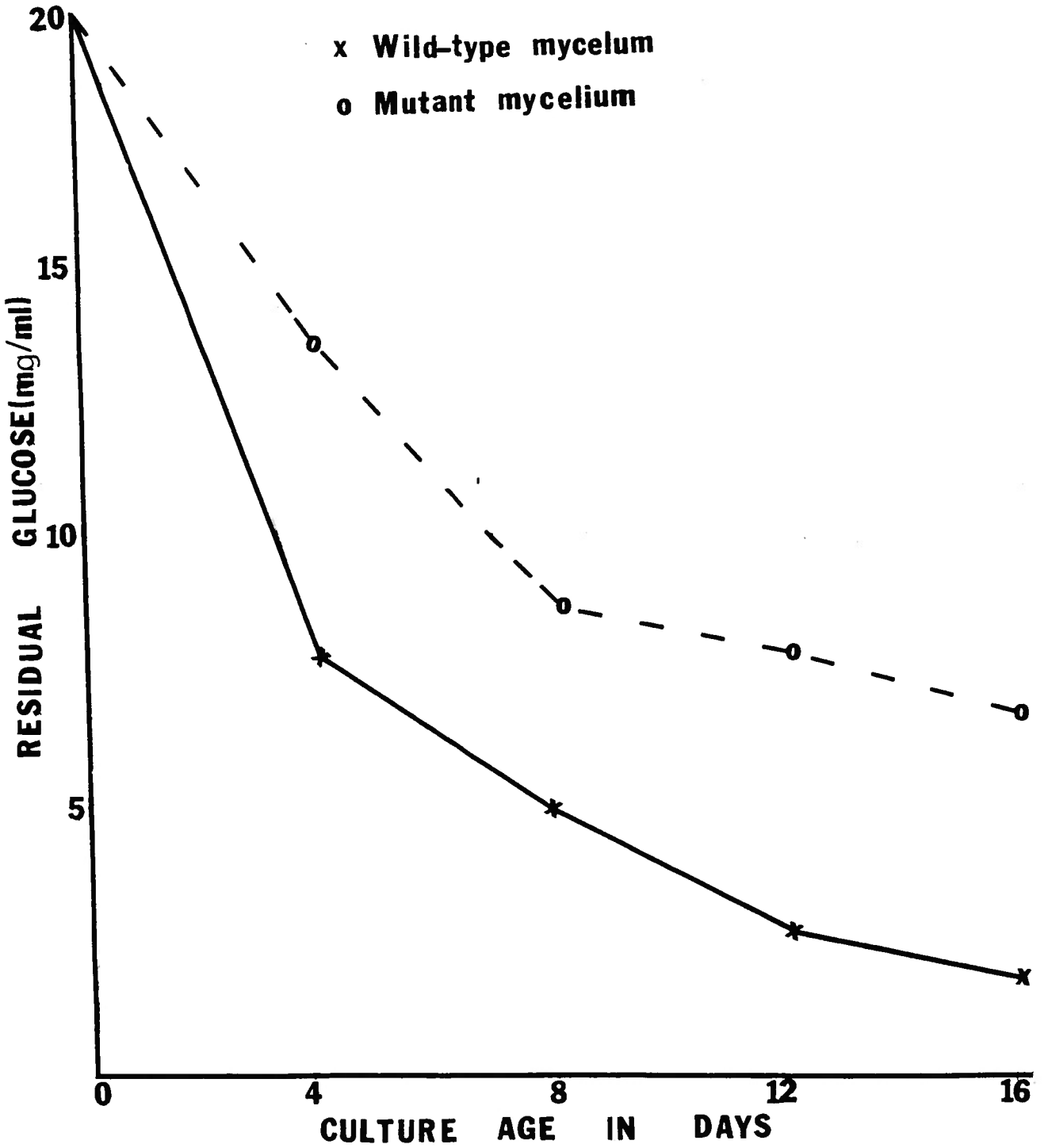
x Wild-type mycelum
o Mutant mycelium
RESIDUAL GLUCOSE(mg/ml)
20
15
10
5
0
4
8
12
16
CULTURE AGE IN DAYS

Fig. 2.

Fig. 2. Residual glucose of wild-type and chocolate mutant in complete liquid medium.

weight and protein content are normally associated with the physiological state of fungal organisms.

The yield of cellular crude protein of the wild-type mycelium was higher at the end of the 12 day growth period than that of the mutant. There were 2.52 mg protein/mg dry weight of mycelium in wild-type mycelium as compared to 0.49 mg protein/mg dry weight for the mutant mycelium (Table 3). The measurement of the mutant's growth medium showed a drastic increase of protein content to 4.5 mg protein/mg dry weight (74%) whereas in the wild-type growth medium the protein content decrease to 0.60 mg protein/mg dry weight (26%). The protein values are shown graphically in Fig. 3.

S-glucan and R-glucan Contents of Strains 699 and 9004

Soluble (S) and resistant (R) glucans, distinguished by their solubility in alkali (IN KOH) comprise a significant proportion of the cell wall of S. commune (Wessels and Sietsma, 1979). To obtain information about the distribution of the alkali-soluble and insoluble polysaccharides in these cell types, partial hydrolysis of the polysaccharides was carried out.

The S-glucan hydrolysate of the mutant mycelium showed that there was a higher yield, 42.8 mg S-glucan/ml, during a 12 day growth period compared to 36 mg/ml observed in the wild-type (Table 4). The R-glucan hydrolysis of the strains showed a conspicuous difference in the amount of R-glucan in the mycelia of both strains. The hydrolysis of R-glucan

Table 3. Crude protein content of mycelium and growth medium of the strains after 12 days

Sample	Strain	Protein µg/mg dry weight[a]
Mycelium	9004	0.49
"	699	2.52
Complete medium	9004	4.50
"	699	0.60

[a]Average of three experiments

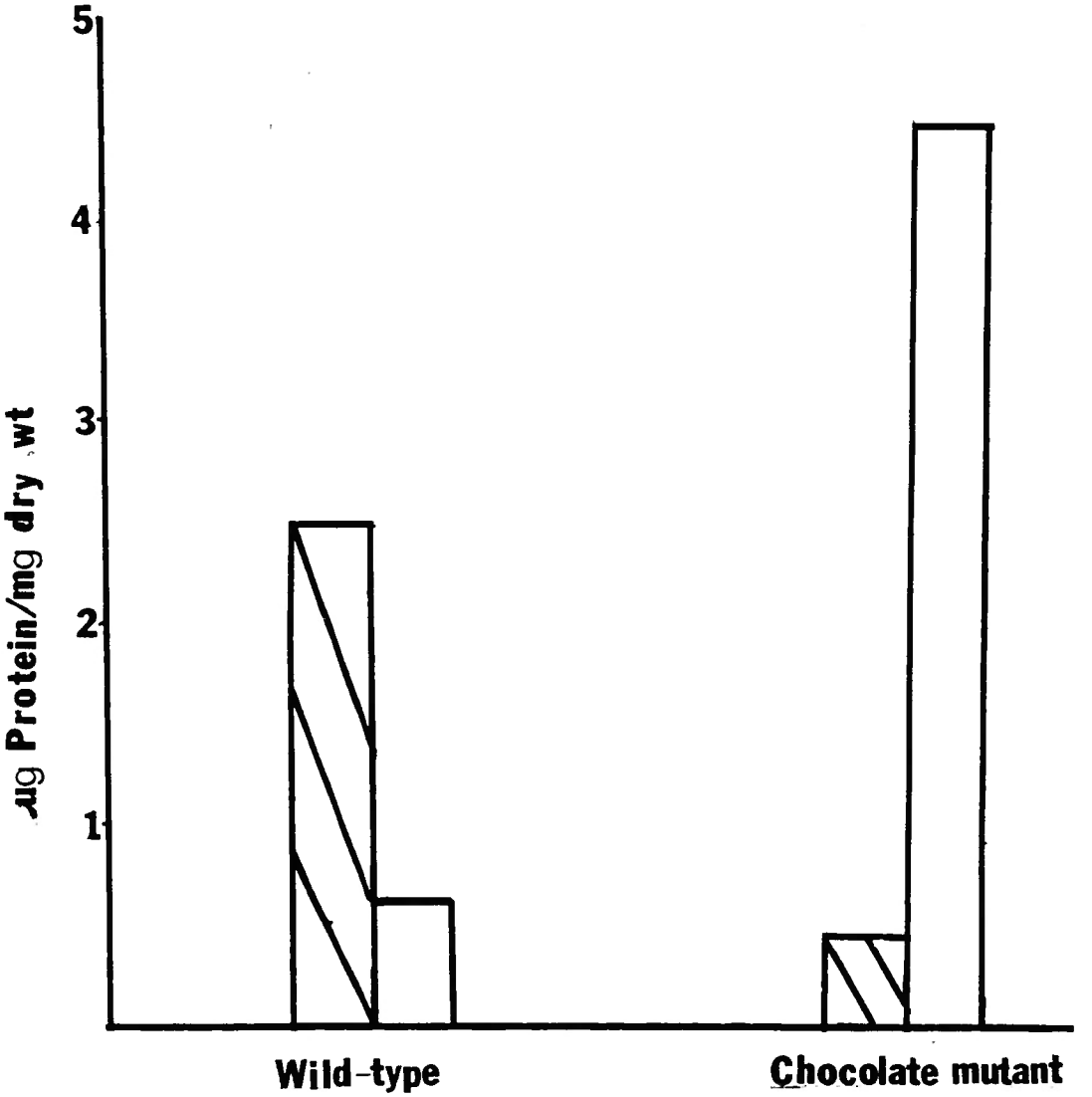

Fig. 3.

Fig. 3. Crude protein content of the cells and medium of wild-type and chocolate mutant. Note cellular crude protein |▨| and extracellular crude protein |▭|.

Table 4. Quantitative distribution of S-glucan, R-glucan and chitin in wild-type and chocolate mutant of S. commune

Strain	S-glucan Glucose mg[a]	R-glucan Glucose mg[a]	S/R ratio	Chitin Glucosamine mg[a]
Chocolate mutant	42.1	29.3	1.43	4.1
Wild-type	36.0	5.9	5.9	22.8

[a]Average of three experiments

showed that the mutant's cell wall yielded 29.3 mg R-glucan/ml whereas that of the wild-type was 5.9 mg/ml.

The S/R ratio obtained for the mutant and wild-type strains are 1.43 and 5.3 respectively. This ratio agrees closely with the result obtained by Wessels (1965). The data show that the S/R ratio of the latter is 4 times higher than the former (Table 4). At the same time, the hydrolysis of strain 9004 showed a yield of 4.1 mg/ml of chitin compared to a higher yield of 22.8 mg/ml of the wild-type mycelium (Table 4).

Incorporation of Uridine Diphosphate-[^{14}C]-N-acetyl-D-glucosamine into Insoluble Chitin

Since chitin is an important component of the cell-wall of basidiomycetes, and since the hydrolysis of chocolate's cell-wall showed a small amount of chitin (4.1 mg/ml; see Table 4), it was decided to determine if there is a possible alteration in the chitin synthsizing enzyme system by observing the incorporation of uridine diphosphate [^{14}C]-N-acetyl-D-glucosamine (UDP-[^{14}C]GlcNAc) into insoluble chitin. The incubation of UDP-[^{14}C]GlcNAc with a cell-free extract of the wild-type revealed a comparatively high incorporation of the label during a 2 hr period. The wild-type strain yielded 426 cpm compared to 11 cpm for chocolate (Table 5). The above results show that there is about a seven-fold increase in the incorporation of UDP-[^{14}C]GlcNAc into insoluble chitin of strain 699.

Table 5. Incorporation of uridine diphosphate-[^{14}C]-N-acetyl-glucosamine into insoluble chitin and the cell wall of wild-type and chocolate mutant of S. commune

Insoluble chitin		Cell wall	
Strain	cpm/mg dry weight	Living cpm/mg dry weight	Heat Shocked cpm/mg dry weight
9004	11	6	3
699	426	10	4

Incorporation of Uridine Diphosphate-[^{14}C]-N-acetyl-D-glucosamine into the Cell Walls of Strains 699 and 9004

In order to study the incorporation of UDP[^{14}C]GlcNAc into fungal cell wall, it was decided to carry out an incorporation study of UDP-[^{14}C]GlcNAc into the cell walls of the chocolate mutant and the wild-type. The results showed rather low cpm that was comparable to the background count (Table 5).

Phase Contrast Microscopy

Strain 9004 grown on semi-solid complete medium showed submerged and uniform growth (Fig. 4). On the other hand, the wild-type is characterized by white hyphae and uniform growth (Fig. 5). The hyphae of the wild-type exhibit cross-wall. This is a major morphological characteristic of the higher basidiomycetes (Fig. 6) whereas the mutant shows a lack of cross wall (Fig. 7). Nuclei are observed along the subapical regions of the hyphae. As the hyphae of strain 699 elongated, branching occurred at an angle 45° with the parent hypha (Fig. 8), while that of strain 9004 occurs at right angles with the parent hypha (Fig. 9), thus giving a reticulated appearance to the mycelium.

Surface Views of the Wild-type Strain 699 and Mutant Strain 9004

Because of the accumulation of exudate, high protein content of medium, and cell wall alteration due to low chitin level, the surface was examined with the scanning electron microscope. The SEM view of both strains shows that there

Fig. 4. Chocolate mutant grown on complete agar medium. Note brownish aerial hyphae produced by the mutant.

Fig. 5. Wild-type grown on complete agar medium. Note space and whitish hyphae produced by this strain.

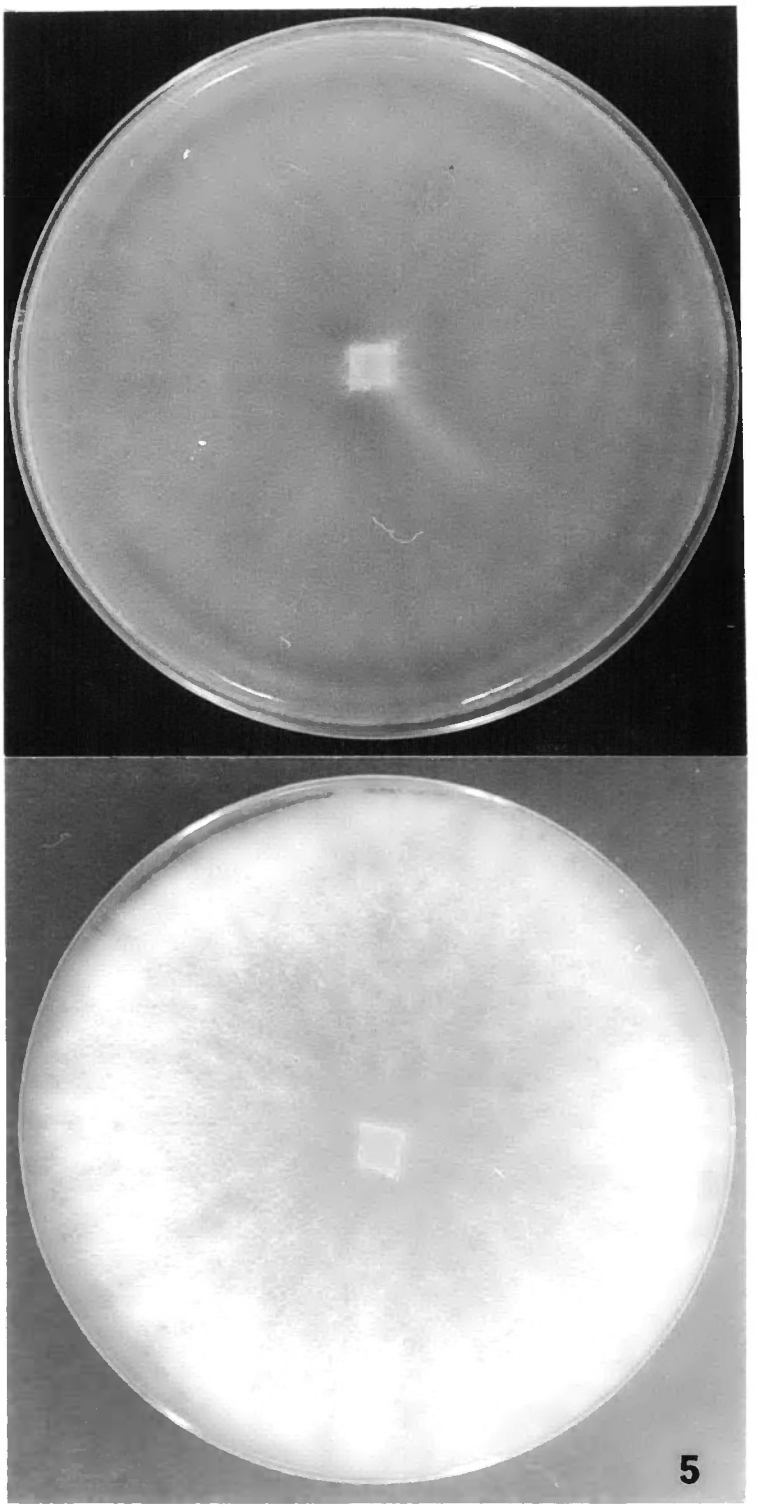
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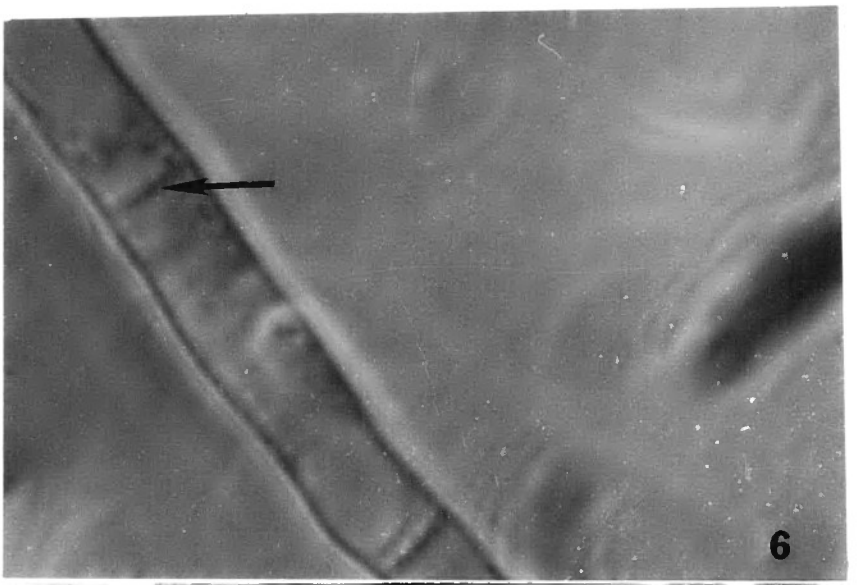
Fig. 6. Wild-type mycelium grown on cellophane membrane. Note cross wall (arrow) produced by the hypha. x 600.

Fig. 7. Chocolate mutant grown on cellophane membrane. Note nonseptate hypha with nucleus (n), vacuole (v). x 600.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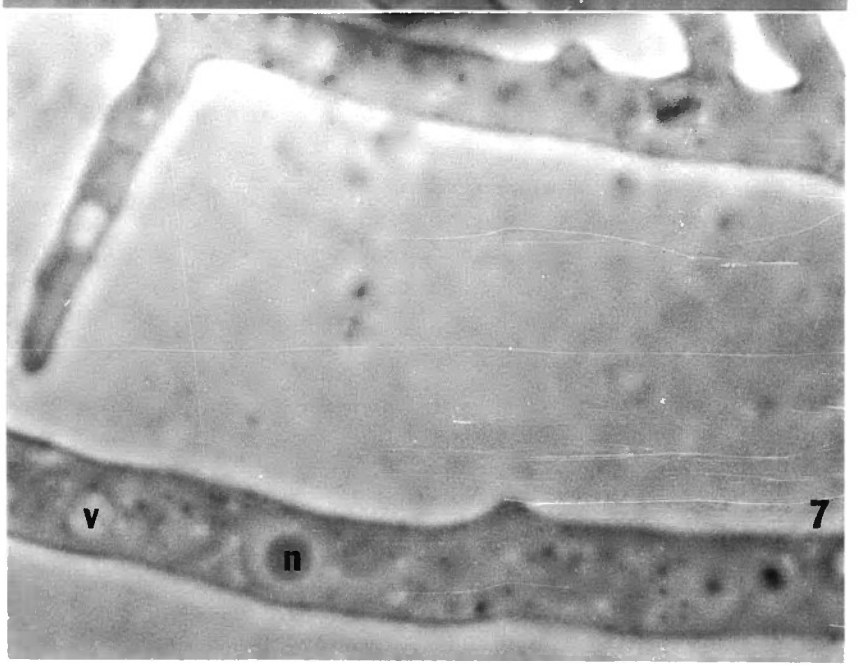
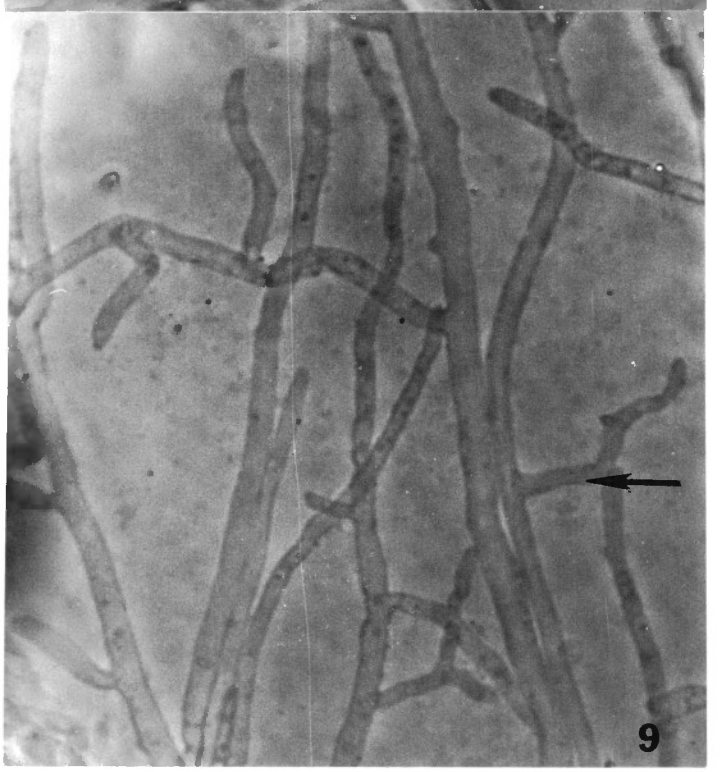

v
n
7

Fig. 8. Microscopic view of wild-type hyphae grown on cellophane membrane. Note hypha branching at 45° angle (arrow). x 400.

Fig. 9. Chocolate mutant grown on cellophane membrane. Note hyphae branching at right angle (arrow). x 400.

8



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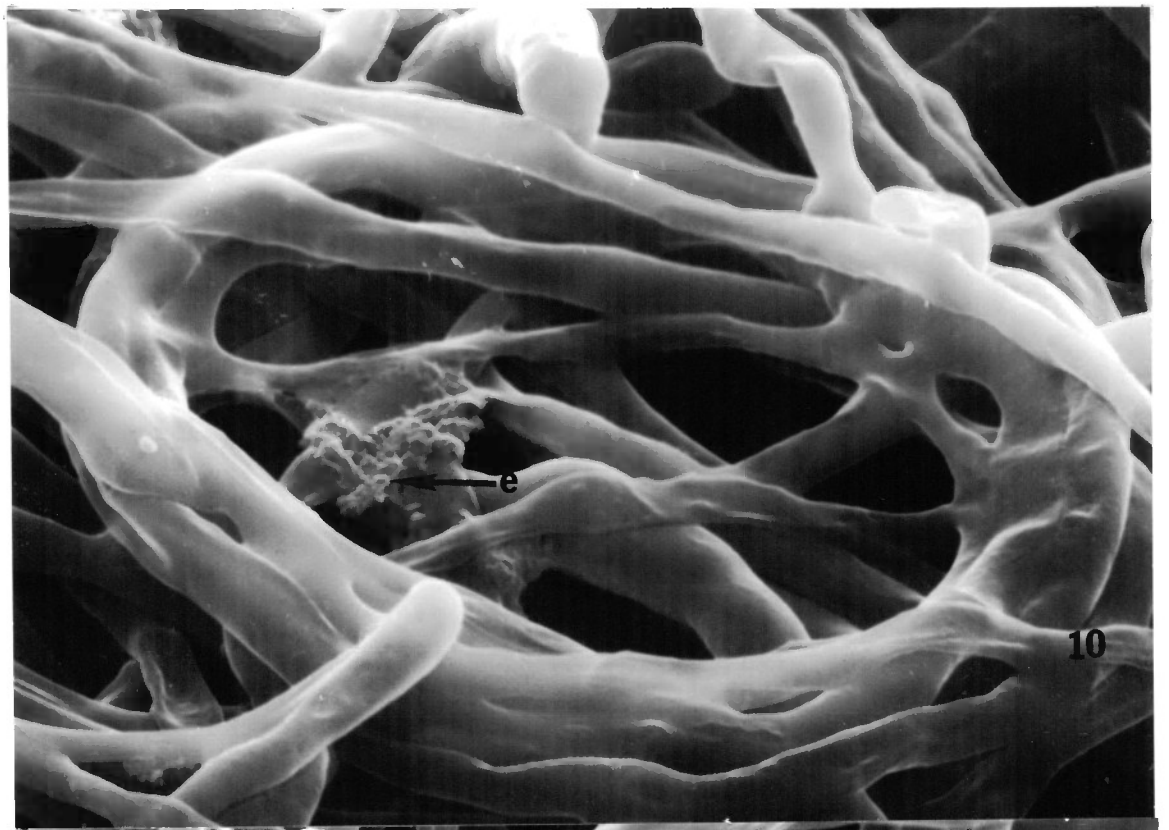
were significant differences in the structural features. The surface of strain 9004 mycelia exhibited some cytoplasmic materials when observed under SEM (Fig. 10). The chocolate mutant viewed with SEM showed that the hyphae often grow in a ring form. There is morphological evidence that some of the hyphae developed pouchlike structures which can be observed in scanning electron micrographs (Figs. 10-15). Further, the hyphal walls of the mutant appear smooth (Fig. 10). These structures are somewhat variable in shape, but most are rather spherical. The surface of each appears smooth when viewed with SEM. The structure first appears as a small pouch on the hyphal wall (Fig. 12). As the young structure develops, it enlarges in size (Fig. 13). Next, the pouch-like structure became spherical and matured, which subsequently ruptured and eventually secreted cytoplasmic materials (Fig. 14, 15). On the other hand, such exudate material is not observed on the surface views of the wild-type's hyphal wall. The SEM micrograph also shows that the hyphae of strain 699 are rough, parallel to one another and lack pouch-like structures (Fig. 16).

Transmission Electron Microscopy

The most obvious features visible in thin sections of strain 9004 are shown in Figures 17-30. A cytoplasmic enriched hyphal region of chocolate mutant exhibiting the following numerous vesicles, vacuoles, mitochondria, and dense ribosomal population were observed in Figure 17.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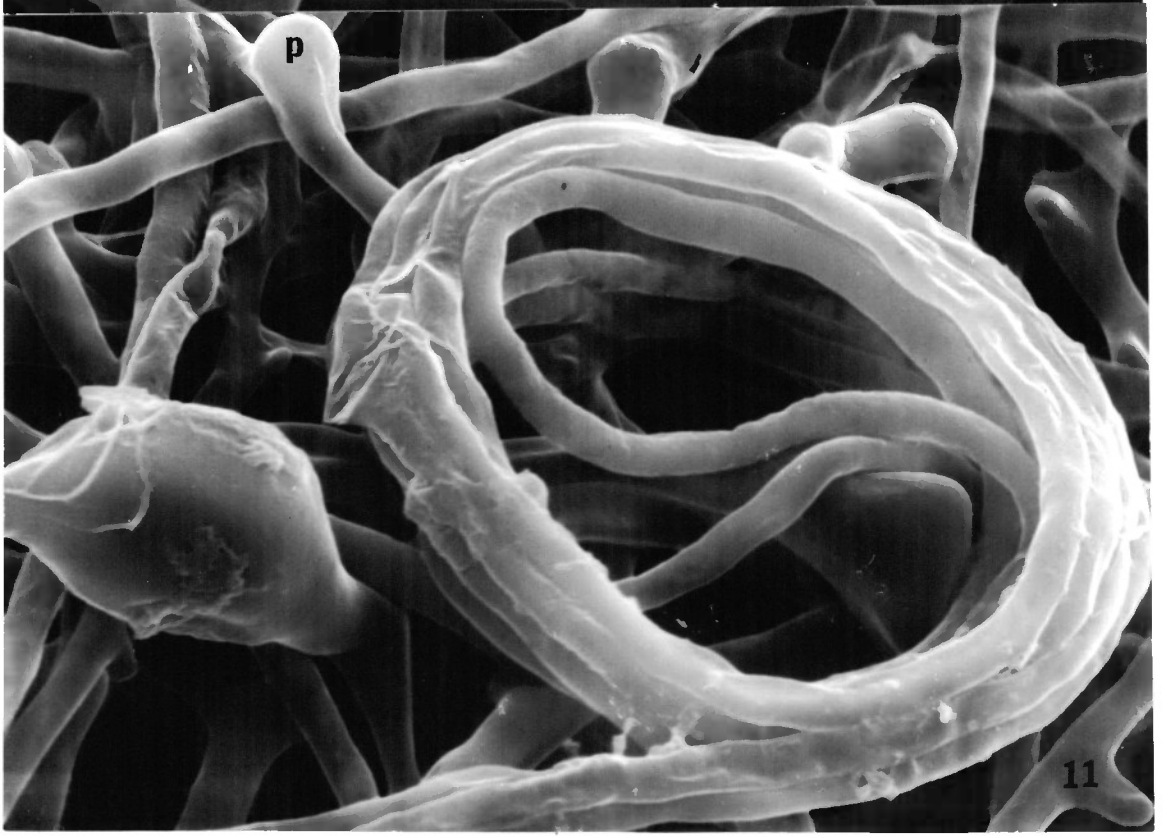
p
11

Fig. 12. SEM of chocolate mutant at higher magnification illustrating various sizes of pouch-like structures. Note small pouch-like (arrow) structure on the hypha wall. x 2,400.

Fig. 13. SEM of a portion of an intact developing pouch-like (arrow) structure. x 2,400.

Fig. 14. SEM of pouch-like (arrow) structure. Note the smooth and spherical morphology. x 2,400.

Fig. 15. SEM of ruptured pouch-like structure. Note cytoplasmic materials. x 2,400.

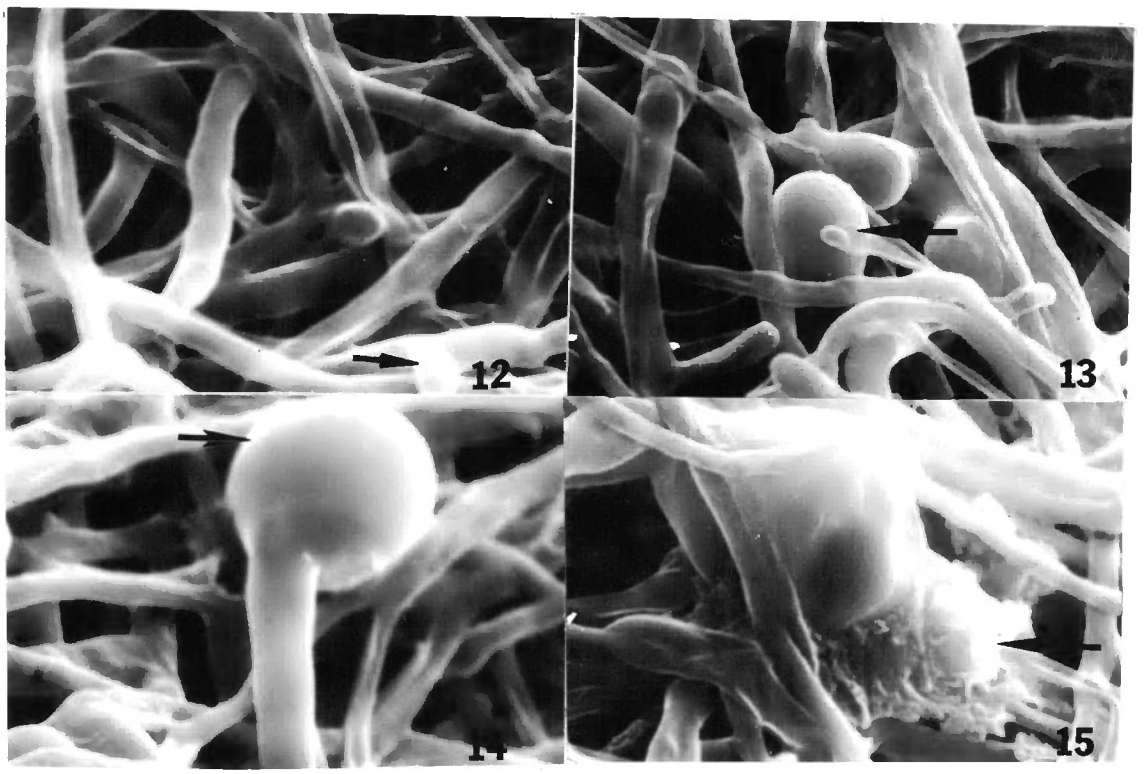
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Fig. 16. SEM of wild-type hyphae. Note lack of pouch-like structures on the hyphae and the roughness of hyphal surface. x 2,400.

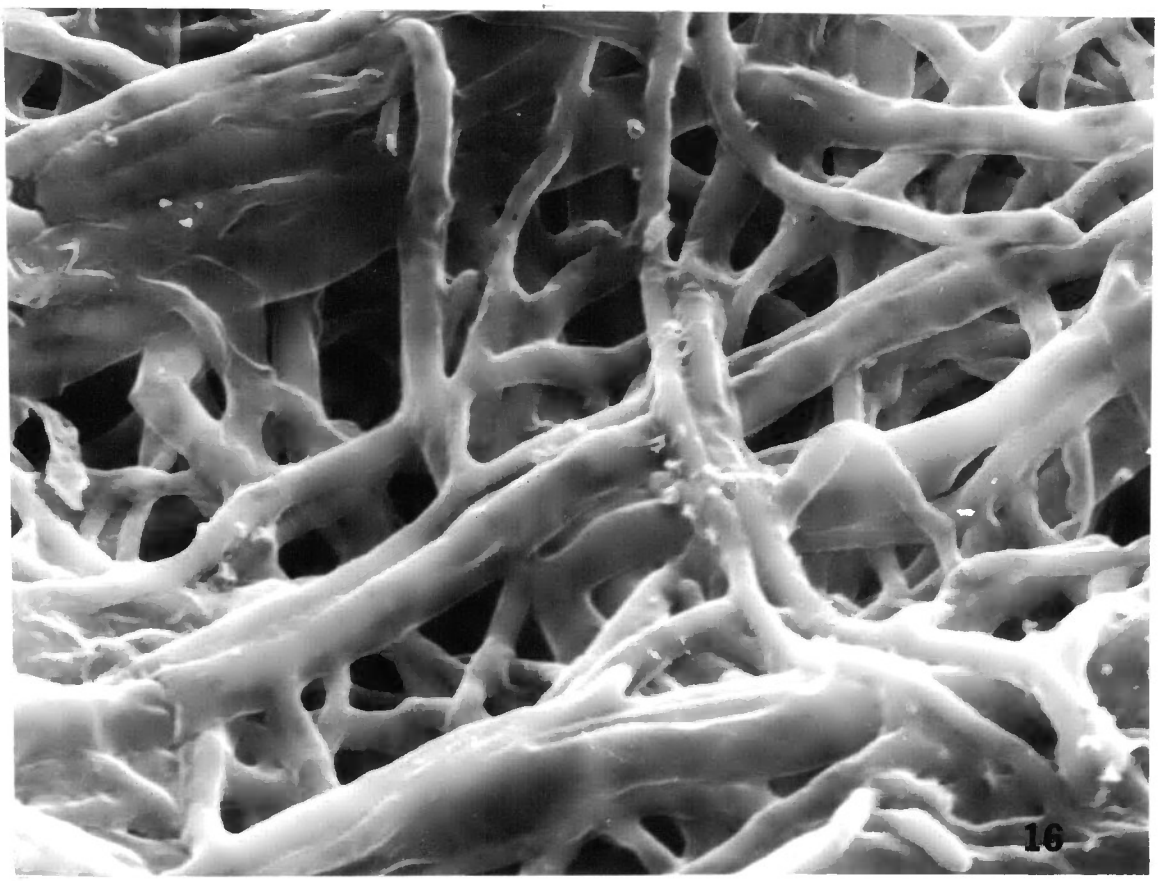
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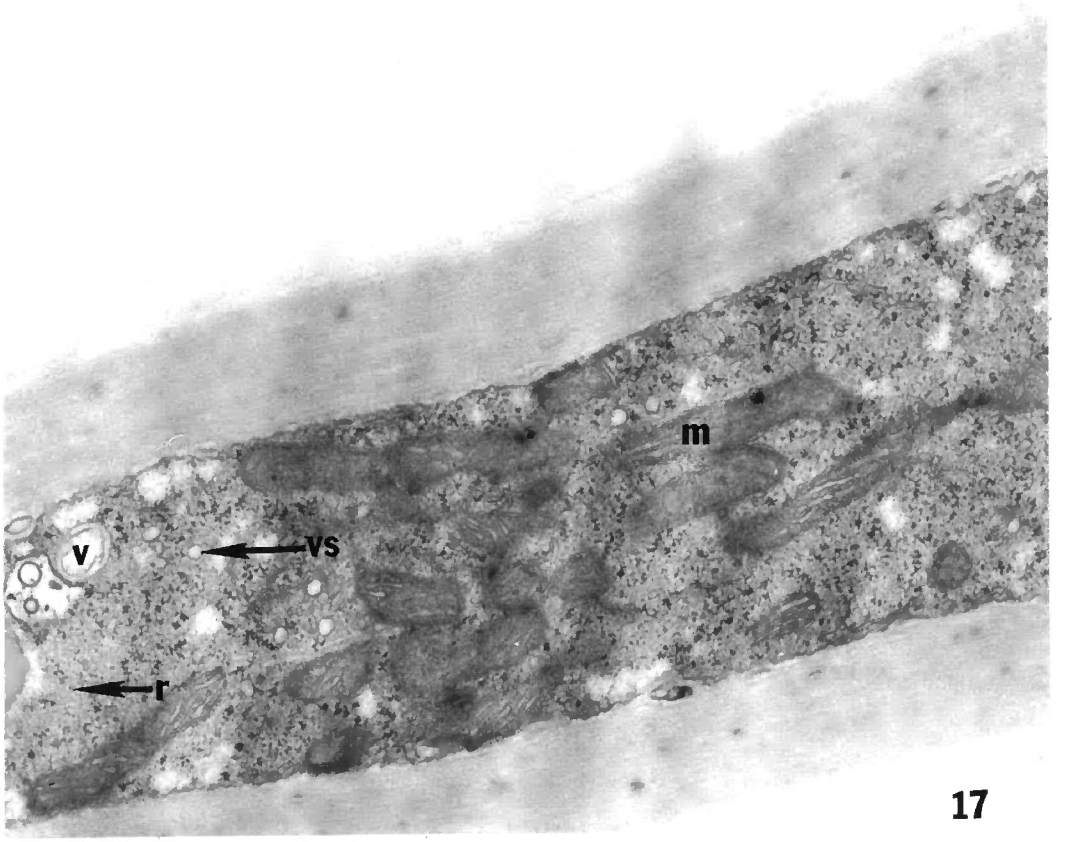
Fig. 17. TEM of cytoplasmic enriched region of chocolate mutant illustrating mitochondria (m), vacuoles, (v), and dense ribosomal (r) population. Note numerous vesicles (vs). x 51,000.



17

In the subapical area the vacuoles contained more elaborate cytoplasmic materials (Fig. 18). The large granular masses were observed within the vacuoles that were associated with this area. Also observed in this region were vacuoles, lipid, endoplasmic reticulum, microtubules and intrusion in the hyphae.

There was a reduction in the concentration of ribosomes in subapical hyphal regions which are near the apex (Fig. 19). Altered mitochondria and nucleus were observed in this region. The mitochondria were altered in that their structure included lipid-like granules. There were also different shaped mitochondria and their structure did not include conspicuous outer mitochondrial membranes despite the presence of rather well defined cristeal membranes.

In order to compare the cell wall structure of the nonseptate mutant with that of the wild-type strain, the hyphal wall of strain 699 was observed as has been reported by Sietsma and Wessels (1977). TEM observations of the wild-type strain show that the cell wall is normal and rigid (Figs. 20, 21). In addition, the micrograph also illustrates a well developed septum (Fig. 22) which is one of the major characteristics of basidiomycetes, while the cell wall of the mutant consists of longitudinal layers of fibers (Fig. 19). Also the cell wall of the mutant is wider than that of the wild-type strain. Intrusions were observed in the mutant strain (Figs. 23, 24). Another observation is

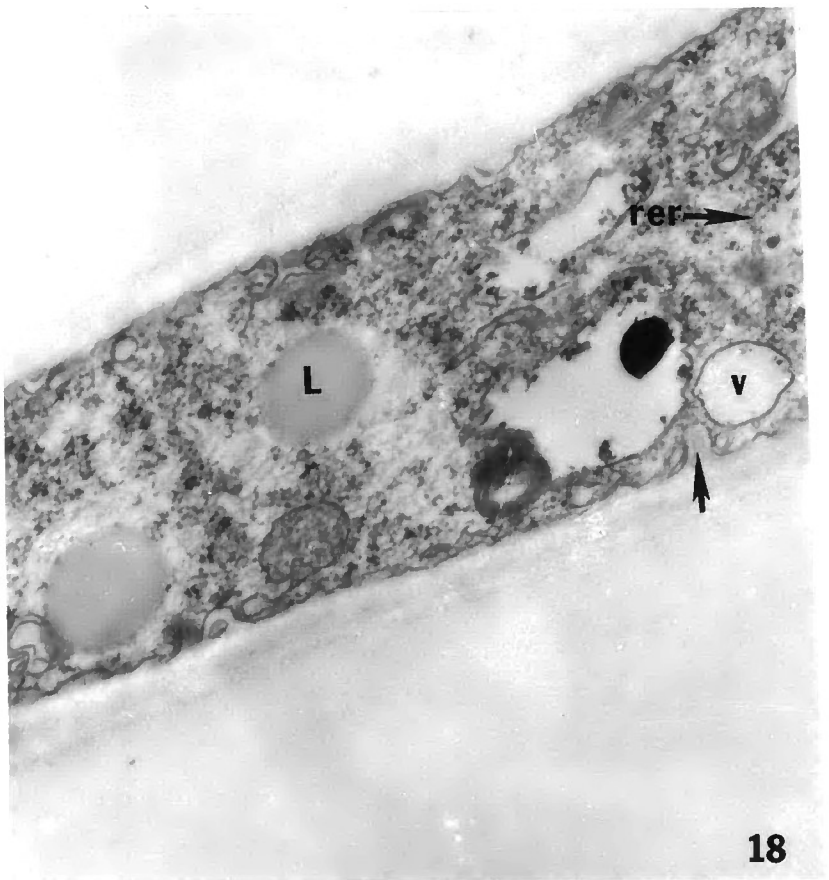

rer
L
v
18

Fig. 19. TEM of a portion of an intact hypha of chocolate mutant illustrating nucleus (N), mitochondria (m) and part of the cell membrane in contact with vacuole (arrow). Note the width of the cell wall (cw) and longitudinal layers of fiber (arrow), and altered mitochondria (arrow). x 3,600.

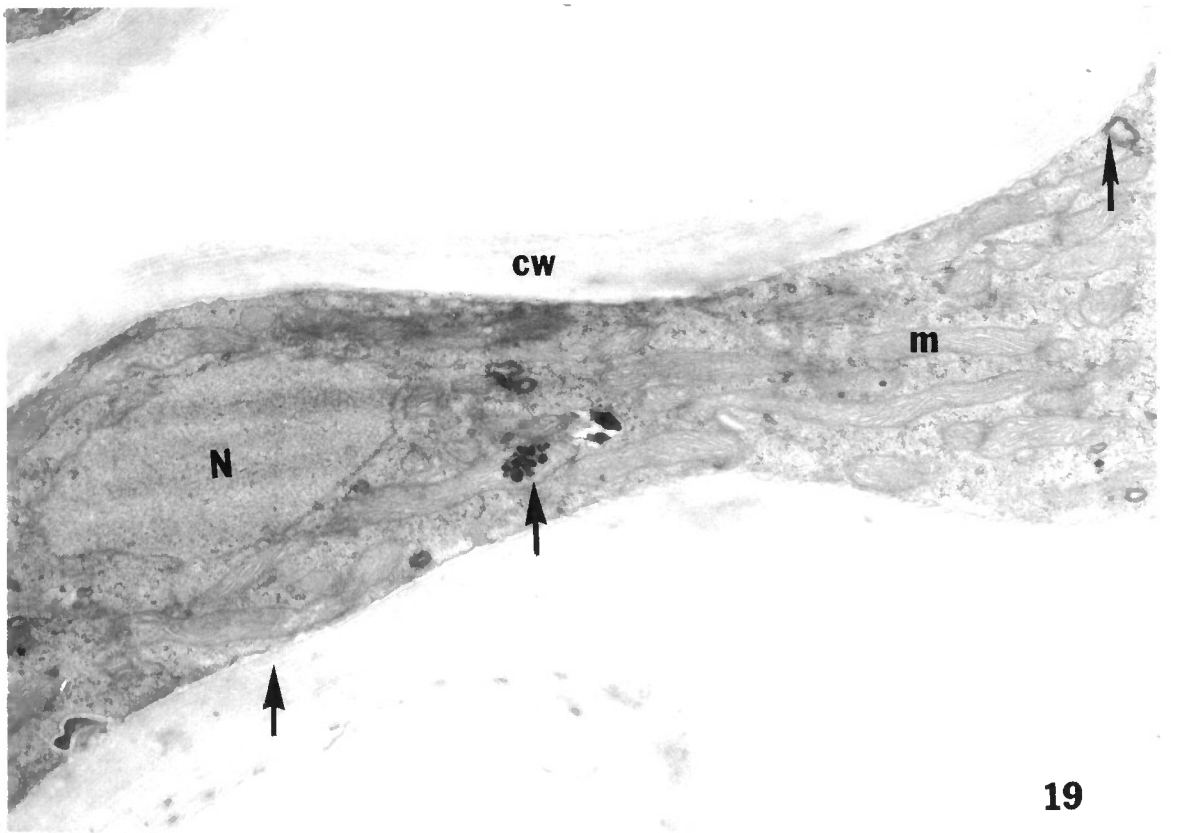


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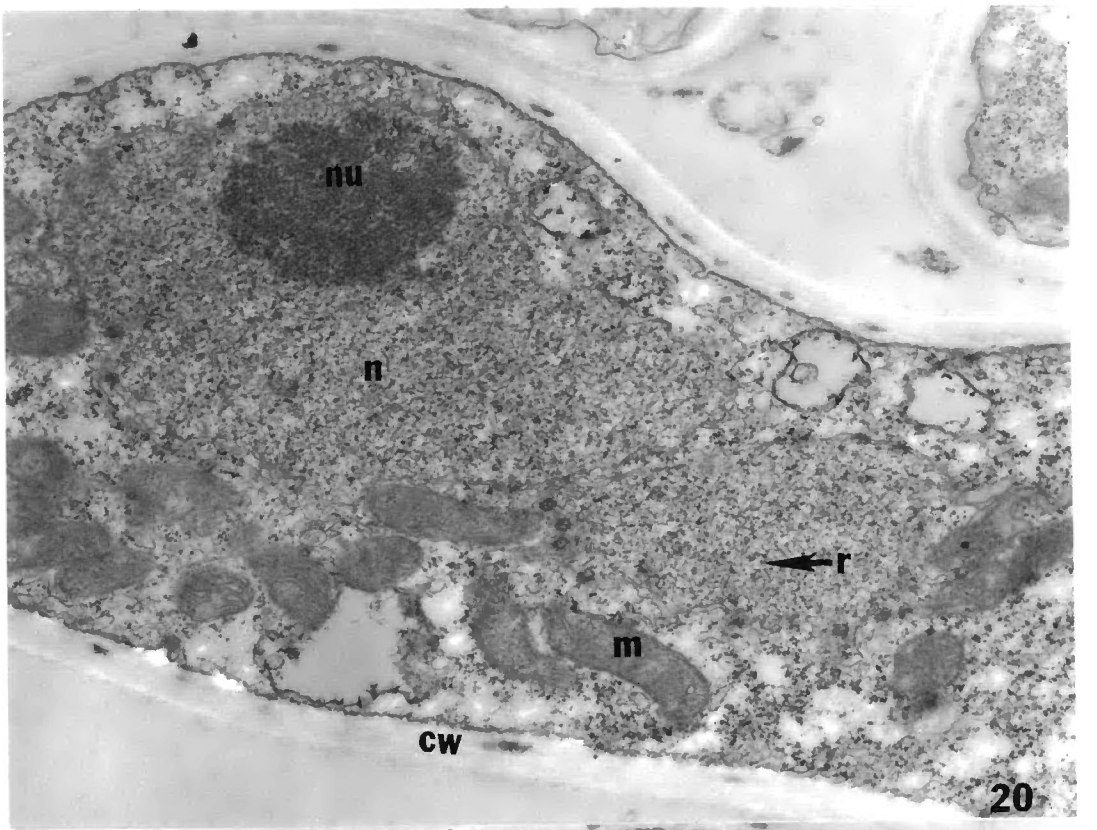
Fig. 20. TEM of wild-type illustrating nucleus (n), nucleolus (nu), mitochondria (m) and ribosomes (r). Note width of cell wall (cw). x 36,000.

Fig. 21. TEM of subapical region of wild-type showing lipid (L), mitochondria (m) and vacuole (v). Note cell wall (cw) rigidity. x 36,000.



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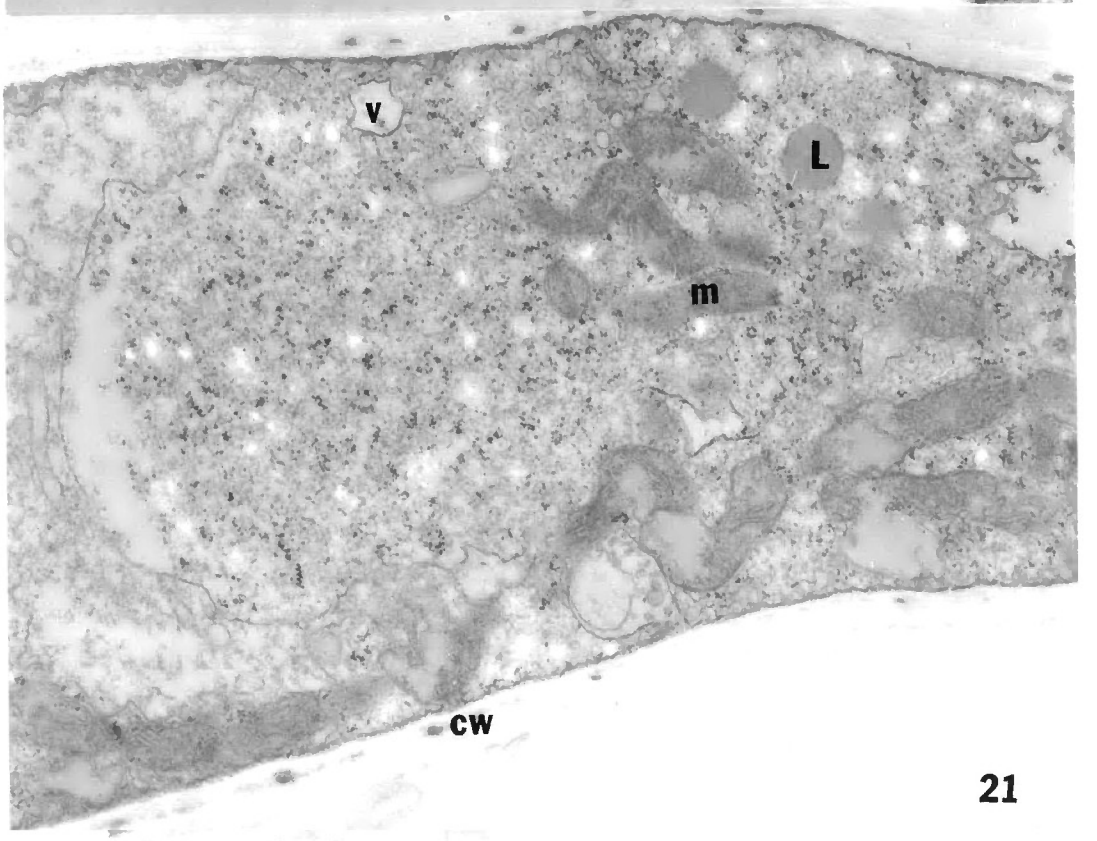

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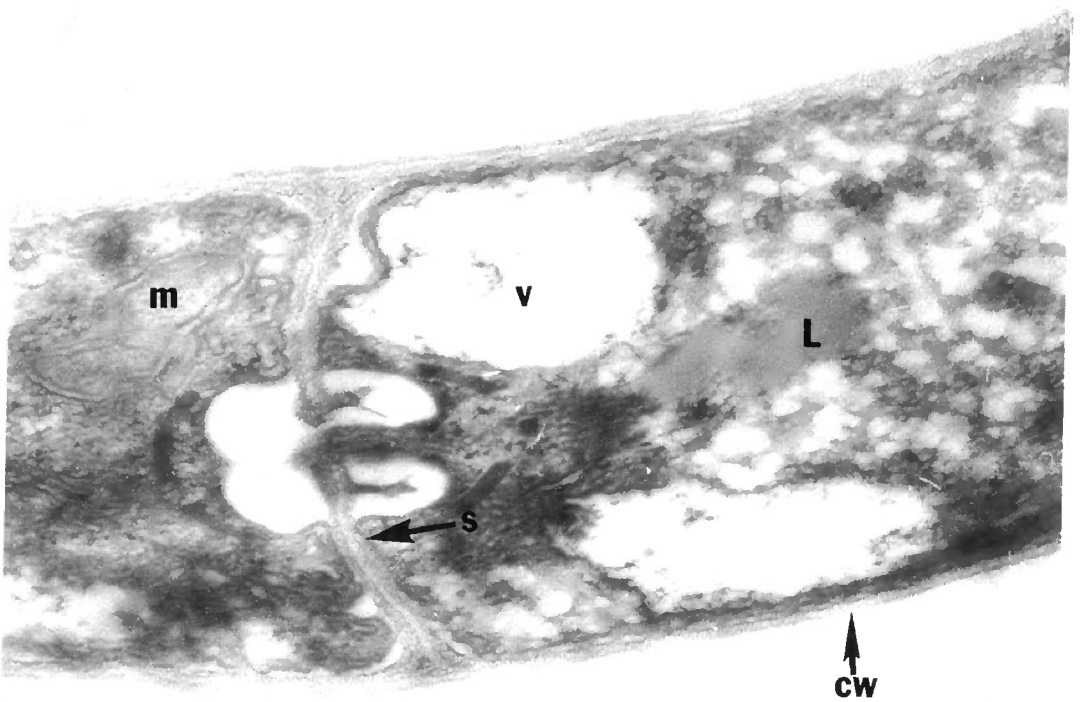
Fig. 22. TEM of wild-type hypha illustrating septum (s), mitochondria (m), vacuole (v), lipid (L). Note the width of the cell wall (cw). x 24,000.

Fig. 23. TEM of chocolate mutant hypha showing cell wall intrusion (arrow) and cell wall (cw). x 36,000.



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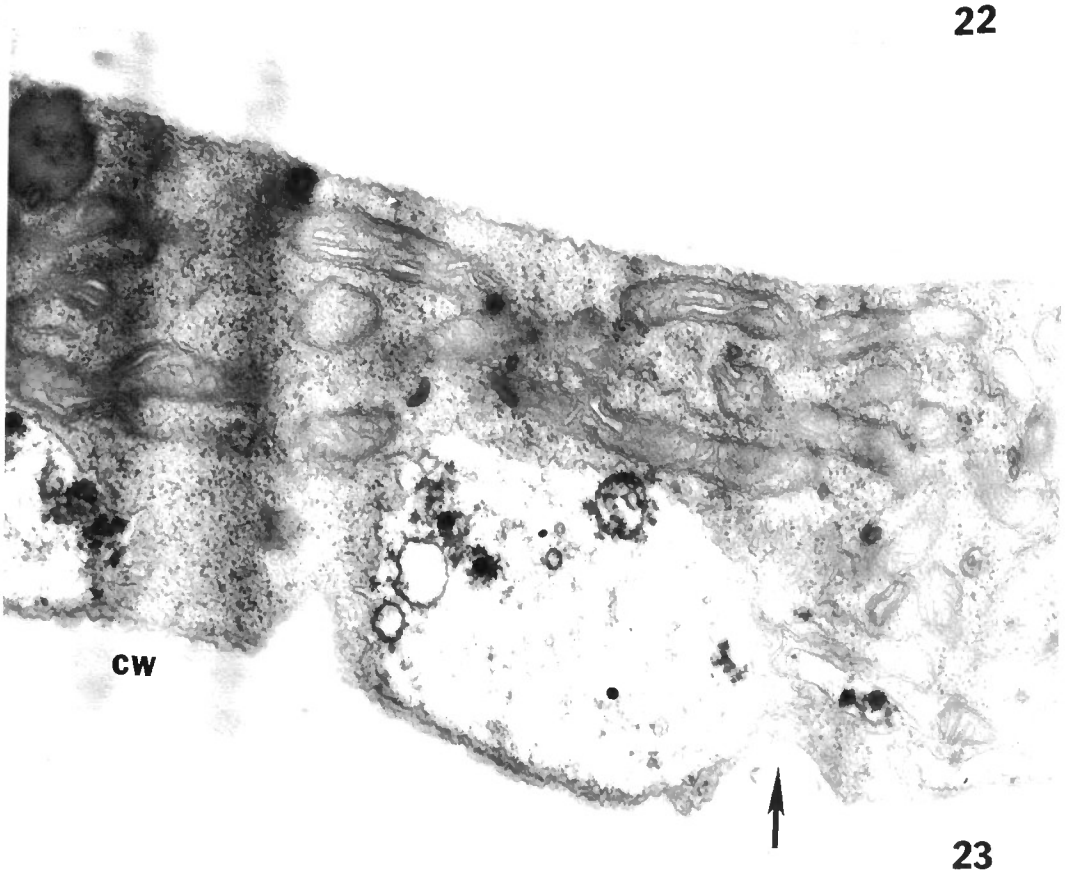
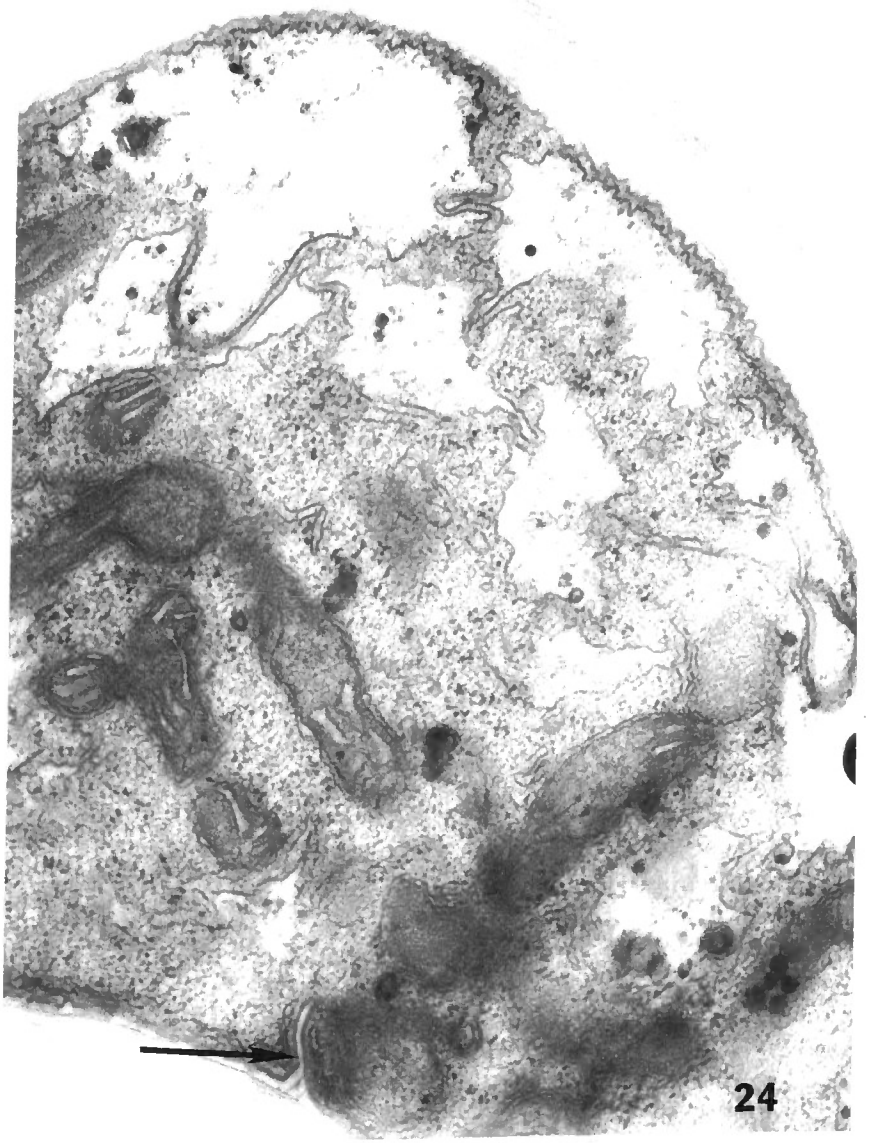


23

Fig. 24. TEM of a portion of chocolate mutant hypha showing pronounced intrusion (arrow). Note cell membrane irregularities (arrow). x 36,000.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that vacuoles are in contact with the cell membrane and in some regions of the cell wall an intrusion was observed (Fig. 25). The ultrastructure of this region (Fig. 26) revealed vacuoles and lipid which are filled with cytoplasmic materials. This region of the hypha exhibited a large vacuole (Fig. 27). Both vesiculated mitochondria and club-shaped mitochondria with parallel cristae were also observed. observed. The cytoplasm of the hyphal region was dense with ribosomes (Fig. 28), contained many vesicles just beyond the mitochondria, endoplasmic reticulum and numerous mitochondria with clearly discernible cristae. A well-defined cell membrane was observed in this region (Figs. 27, 28).

The electron microscope studies revealed hyphal regions with two nuclear profiles (Fig. 29). The nucleolus, located at one side of the nucleus, was distinct and displayed electron transparent "spurs." Mitochondria were observed around the nuclei. TEM observation of chocolate mutant also illustrates extracellular exudate which was dispersed throughout this area (Fig. 30).

Fig. 25. TEM of chocolate mutant showing nucleus (n) and a series of vacuoles (v) in contact with the cell membrane. Note intrusion in the mutant hypha. x 24,000.

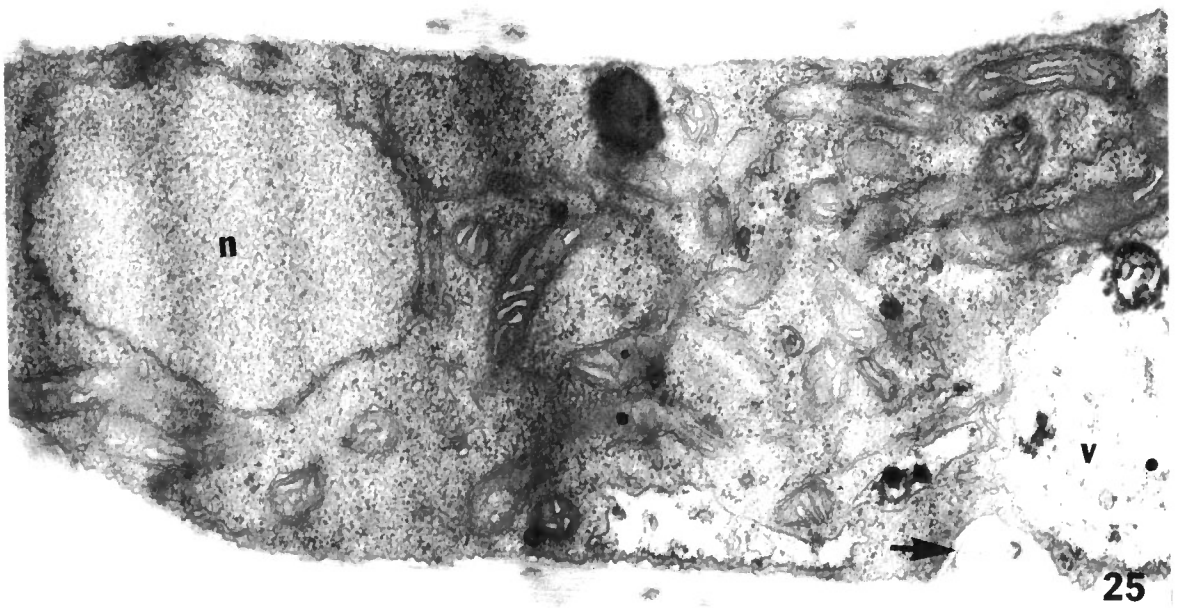
n
v
25

Fig. 26. TEM of chocolate mutant with various shapes of mitochondria (m) and vacuoles containing cytoplasmic material (v). x 51,000.

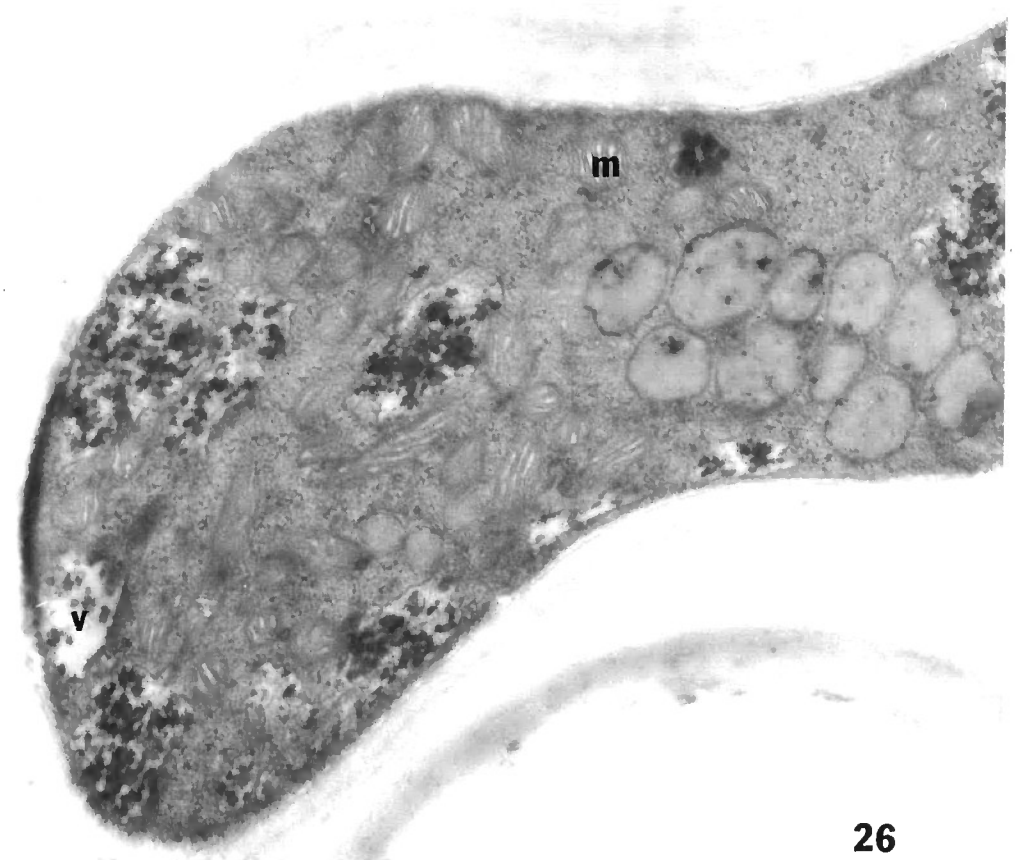

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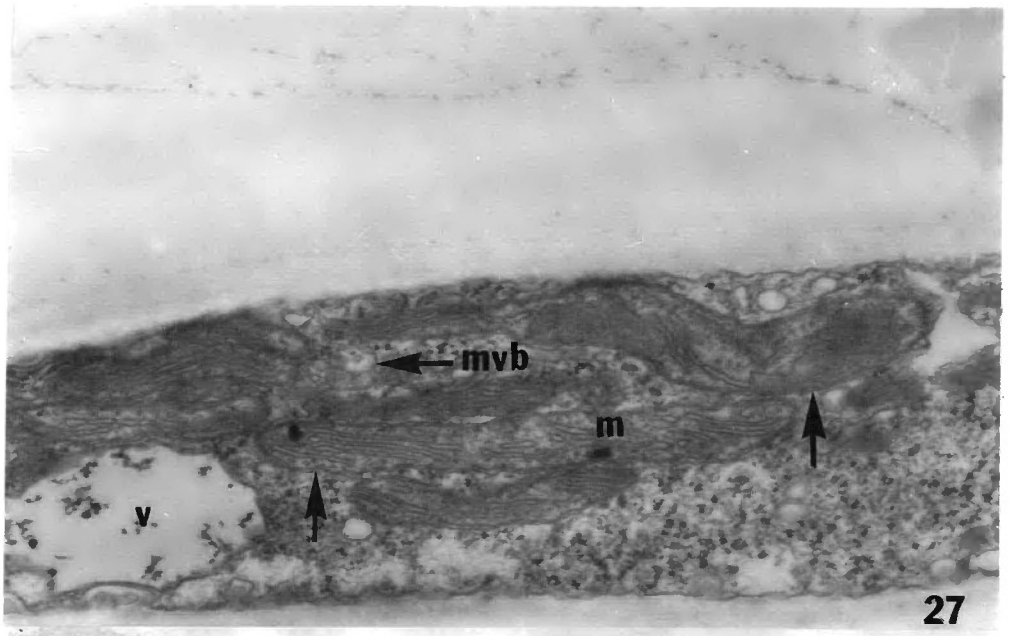
Fig. 27. TEM of chocolate mutant hypha exhibiting mitochondria (m) and multivesicular bodies (mvb). Note series of vacuoles in contact with the cell membrane (arrow). x 51,000.

Fig. 28. TEM of chocolate mutant hypha with increasing frequency of ribosomes (r), different shapes of mitochondria (m) and rough endoplasmic reticulum (rer). Note irregularity of the cell membrane (cm) and the width of the cell wall (cw). x 51,000.


mvb
m
v
27

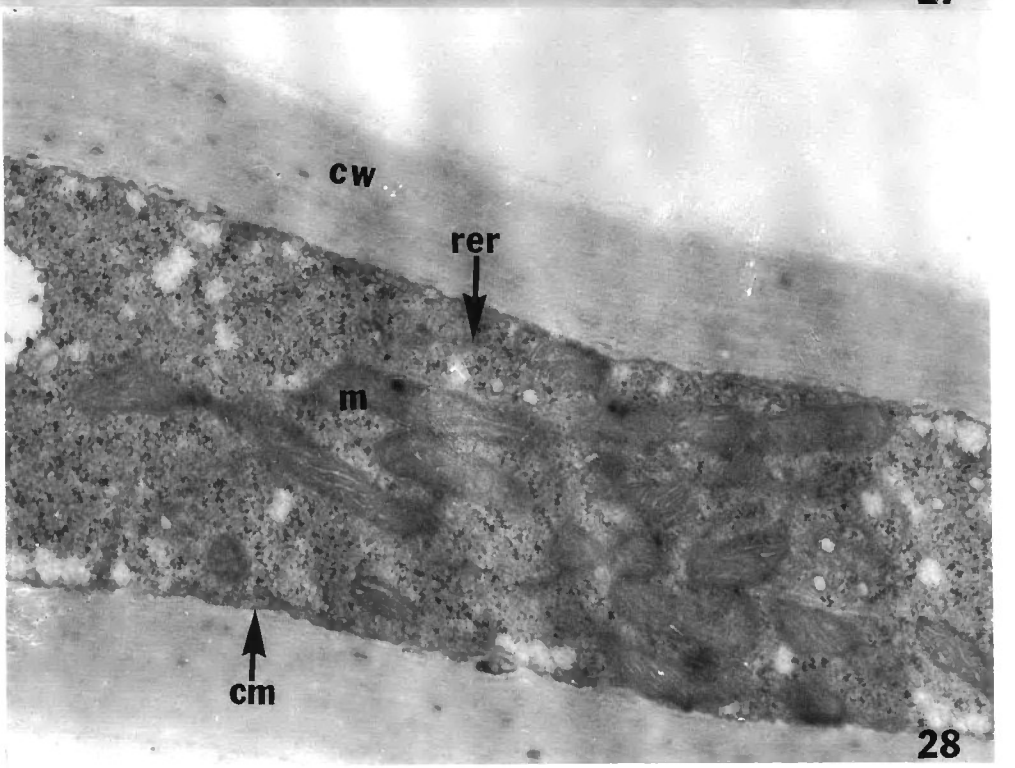

cw
rer
m
cm
28

Fig. 29. TEM of chocolate mutant showing nuclear profiles (n1, n2). Note nucleus with highly granular nucleolus (Nu) and granularity of the nucleoplasm. x 51,000.

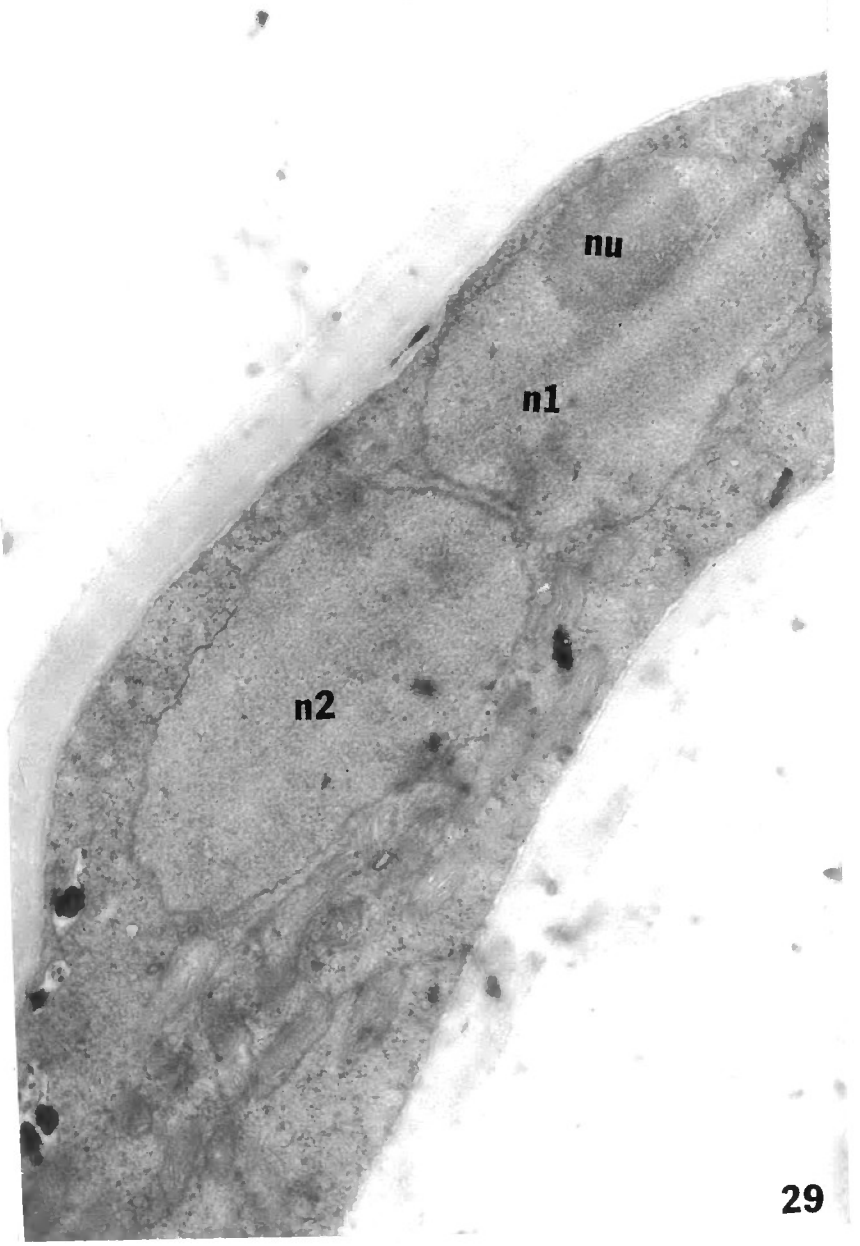


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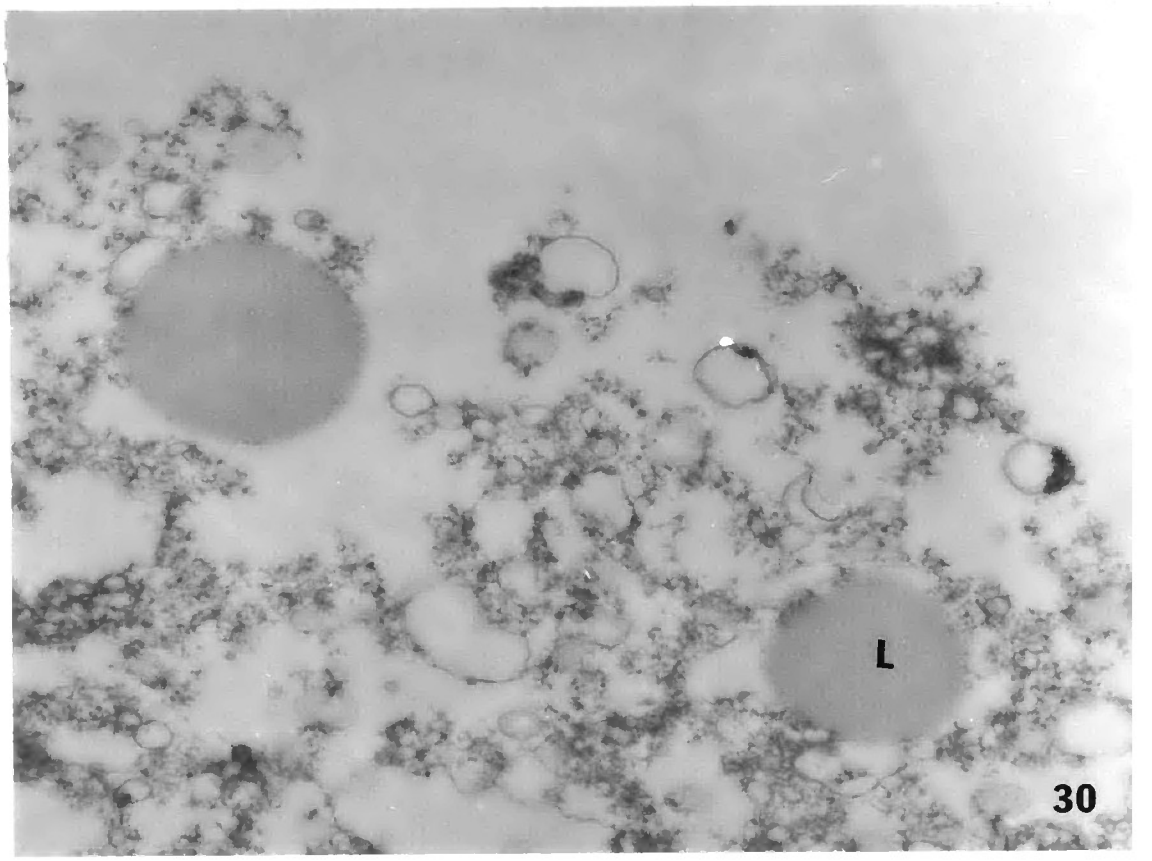
Fig. 30. TEM showing extracellular exudate and lipid bodies (L) outside the hyphal wall. x 36,000.


L
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CHAPTER V

DISCUSSION

The results of this research clearly indicate that the nonseptate condition of the chocolate mutant is related to alterations in cell wall structure and associated metabolic activity. The fact that chitin was not detected in the cell wall of the mutant, suggests that there is a lack of ability to either incorporate precursors such as UPD-GlcNAc into the cell wall or synthesize the necessary cell wall precursors (Keller and Cabib,1971). In their studies, they observed that developing yeast, which are devoid of chitin did not exhibit the presence of the enzyme chitin synthetase which is responsible for the synthesis of chitin. Based on observations of the N-acetyl-D-glucosamine assay and lack of UDP-GlcNAc incorporation, it seems as though the nonseptate characteristic of strain 9004 is due to its inability to synthesize chitin.

The increase in R-glucan content observed in the mutant strain (Wessels, 1965; Wessels, 1969) has led Sietsma and Wessels (1979) to suggest that the poor growth of these hyphae could be due to lytic activity directed toward its cell wall material. Lack of breakdown of R-glucan revealed by the present study on the chocolate mutant hyphae suggests that in addition to the general lytic activity directed

towards cell wall material, there may be a lack of degradation and resynthesis during the development of the mutant hyphae.

Scanning electron micrographs of the chocolate mutant show extra hyphal accumulation which suggests that the hyphal walls of strain 9004 were excreting some of their cytoplasmic materials. This suggests that the higher crude protein contents detected in the growth medium of strain 9004 may have resulted from the excretion of such cytoplasmic materials. The observation of cytoplasmic materials and high protein content in the growth medium led Lahti and Raudaskoski (1983) to suggest that these could be due to either lytic activity directed towards the cell wall or that the protein synthesized by the B mutant is not incorporated into the hyphal cell wall. Furthermore, the observation of cytoplasmic materials suggests that there are possible alterations on the hyphal wall which allow certan cellular elements to escape the hyphal wall. Although the apparent correlation between cell wall structure, extracellular globules and high protein content in growth medium cannot be adequately explained at this time, it is possible that a necessary component or components of the cell wall is not produced by the chocolate mutant, or is produced but could not be incorporated into the cell wall.

The increased frequency of vacuoles in mutant hyphae can hardly be an artifact caused by fixation, as frequently

suggested, since the wild-type strain and mutant strain in this study were cultured and fixed for the TEM or ultrastructural investigation under the same experimental condition, and vacuoles did not occur in the wild-type hyphae. This indicates that the occurrence of the vacuoles was related to the mutant in the chocolate strain 9004. Moreover, the apical region of the mutant hyphae never showed such extensive vacuolation as did the subapical region of these hyphae.

The lack of septa, which is observed in the mutant strain, allows extensive migration of nuclei (Ehrlich and McDonough, 1949; Snider and Raper, 1958; Snider, 1968; Niederpruem, 1980), mitochondria (Watrud and Ellingboe, 1973a, b) and perhaps vacuoles. It can be speculated that such movement might increase accidental contact of vacuoles with hydrolytic enzymes and other cell organelles, thus leading to either breakdown and impaired function of cell organelles such as mitochondria (Lahti and Raudaskoski, 1983). It is also possible that the permeability of the membrane of the cell and the cell wall might be altered, which could lead to swelling and rupture of structures such as cell wall.

The higher accumulation of glucose observed in the growth medium of mimutant led Mitchell et al., (1953) to suggest that the poor growth of the mutant mycelium could be due to malfunction of mitochondria or lack of energy

conservation which reduces assimilation of substrate while leaving oxidative processes apparently unchanged.

The nonseptate condition which occurs continuously in chocolate mutant allows extensive migration of both cytoplasmic materials and organelles. It is speculated that as these cytoplasmic materials move through the hyphae until they come in contact with internal weak area of the cell wall. As a result of exhaust pressure from the cytoplasmic materials, there is occasional swelling of hyphal walls, and bursting or expulsion of cytoplasmic materials through the cell wall with or without intact cytoplasmic organelles. The inability to demonstrate an appreciable amount of chitin in the cell wall strongly suggests that the nonseptate condition and aberrant hyphal morphology may be linked to the mutant's inability to either synthesize or incorporate chitin precursors into the cell wall.

CHAPTER VI

CONCLUSION

This investigation shows that there is lack of septum in the chocolate mutant, which is due to altered cell wall of this organism, resulting in the absence of chitin, since chitin has been shown to be localized in the septum. Evidence of this is provided by lack of incorporation of UDP-[^{14}C]GlcNAc into the cell wall. Also there is chemical evidence that S/R ratio of the chocolate mutant is low; this suggests that there is weakness in the cell wall.

SEM and TEM observations of chocolate mutant suggest that there is alteration or lesion in its cell wall. Since there is correlation between the hyphal wall disintegration and the high extracellular protein content observed in the growth medium.

All the above evidence suggests that the chocolate hyphal wall is weak and this may be affecting the general physiology of the mutant.

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